# Characterisation of Adult Neural Stem/Progenitor Cells in the Murine Hypothalamus

Thesis presented for the degree of PhD
by

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# Acknowledgements

“If the doors of perception were cleansed everything would appear to man as it is, infinite. For man has closed himself up, till he sees all things thro’ narrow chinks of his cavern.”

~William Blake~

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## Boxes

## Tables

## Figures

# Abstract

Recent evidence has shown that adult neurogenesis is sustained in the hypothalamus, a region of the ventral diencephalon that is the central regulator of homeostasis. While studies support a role for adult neurogenesis in energy balance, as yet, the identity of the neural stem/progenitor cell niche remains contested. Tanycyte cells, a unique population to the hypothalamus, present a possible candidate due to their diverse roles, radial-glial like morphology and position adjacent to the 3rd ventricle. Here, I provide in-vivo, in-vitro and ex-vivo data that together support alpha-tanycytes as a neural stem/progenitor cell population.

My studies show that the embryonic neural stem/progenitor characteristics of radial glia, including expression profile, a basal process and an apical primary cilium, are maintained in alpha-tanycytes during adulthood. In addition, alpha-tanycytes are multipotent in-vivo and contribute to the other tanycyte populations, suggesting a lineage relationship of cells within the hypothalamic ventricular zone. A neurosphere assay adds further validity to the idea that there is heterogeneity in progenitor status within tanycyte subpopulations. Furthermore, alpha-tanycytes are responsive to Fgf-signalling in-vivo, a crucial regulator of proliferation and differentiation during embryogenesis, as well as being required for neurosphere formation.

In order to further interrogate alpha-tanycytes, I developed and optimised an organotypic slice culture protocol, a technique that has not yet been used to study hypothalamic neural stem/progenitor cell dynamics. This ex-vivo technique provides a number of advantages including efficiency, low-cost, and amenability to manipulation, while maintaining large parts of the niche. Exogenous addition of pharmacological agonists and inhibitors reveals that alpha-tanycytes undergo Fgf-dependent proliferation in response to physiological stimulation, and implicates a role for the hypothalamic niche in the homeostatic control of stress.

Together, these studies characterise the component cells of the adult hypothalamic neural stem/progenitor cell niche, providing a framework for future research to further explore the heterogeneity and physiological significance of alpha-tanycytes.

# Chapter 1

## Introduction to adult neurogenesis and hypothalamic function

# 1.0 Introduction

My thesis focuses on the identification and characterisation of neural progenitor cells within the adult mouse hypothalamus and their potential function in the organism. This introduction highlights the main features of adult neurogenesis from its discovery in canaries, through our current understanding of mammalian neurogenesis in the adult central nervous system and its physiological implications. I will then discuss the importance of the hypothalamus in maintaining homeostasis and the evidence for a local adult neural stem cell population. Finally, I will outline the specific aims of my study.

Neurogenesis is defined as the production, migration and differentiation of neurons from stem and progenitor cells. Newborn neurons can take a fate choice between apoptosis or integrating to become a functional unit of the nervous system. In mouse the majority of neurogenesis occurs during embryogenesis between embryonic day (E) 12 and early postnatal life, with a peak period at E14 to E15 (Lemke, 2009). Adult neurogenesis can therefore be defined as neurogenesis that takes place in the post-juvenile, adult system, when neural networks are already established but where newborn neurons contribute to remodelling, refinement and replacement.

Santiago Ramon y Cajal, widely thought of as the father of modern neuroscience, pioneered neural studies by defining classifications of neural cells and theorizing how networks function. The detailed histological studies he performed led Ramon y Cajal to describe the adult central nervous system as fixed, a central dogma that was accepted for a century (Colucci-D'Amato *et al.*, 2006).

> “Once the development was ended, the fonts of growth and regeneration of the axons and dendrites dried up irrevocably. In the adult centres, the nerve paths are something fixed, ended, and immutable. Everything may die, nothing may be regenerated. It is for the science of the future to change, if possible, this harsh decree.” -1893

Almost one hundred years later, in 1980, Nottebohm published evidence of newborn neurons in the brain of the adult songbird (Nottebohm, 1980) that slowly began to change the scepticism that had been faced by early advocates of adult neurogenesis, and laid the foundations for the emerging field of adult neurogenesis.

## 1.1 Evidence of neurogenesis in the adult central nervous system

### 1.1.1 Neurogenesis defined in the High Vocal Centre of the adult songbird.

The central nervous system of the bird develops in the same way as all vertebrates, albeit that the organisation of the adult brain does differ from that of mammals. Within the telencephalon of the forebrain exist specific nuclei that are responsible for producing sound, a feature that is of great importance for food-begging in chicks and for identifying mating partners in adulthood. Of interest is the observation that while all songbirds appear to have innate, unlearned vocalisations that do not change, some species of songbird are additionally able to produce many different repeatable units of sound: song syllables (Nottebohm, 2002).

Song-learning in male canaries first caught the attention of scientists when it was discovered that the repertoire of songs that a canary boasts could increase over time. Moreover, when juvenile male canaries were housed with more mature males, the juveniles began to imitate the mature birds' songs. Studies into the nature of this learning proposed that de-novo juvenile/adult song-learning would confer an evolutionary advantage as it could testify to the individual's other attributes, such as intelligence and ability-to-provide; further, imitating song could increase the chance of mating by making a cuckold of rivals (Nottebohm and Liu, 2010). To determine the nuclei responsible for particular features of song-learning, early studies caused lesions to particular regions of the brain in order to dissect out the pathways mediating this phenomenon.

Analysis of the brains of songbirds reveals a hierarchical circuitry and four modules that are required for song (see figure 1.1) (Nottebohm and Liu, 2010; Alvarez-Buylla, 1992). Module 1 consists of brain stem nuclei (TS), controlling the syrinx (the vocal organ in birds) and rate of respiration in order to physically produce sound. Module 2 consists of two premotor telencephalic nuclei, the hyperstriatum ventrale pars caudalis (HVc or high vocal centre) and archipallium (AP), which give instruction to module 1. Module 3, also receives input from the HVc and projects axons from basal ganglia (X) via the thalamus (DLM) and then anterior cortex (LMAN) to module 1. Module 4 relays information from auditory circuits (Aud) about songs produced and those audible in the vicinity. The HVc sits at the centre of this song circuitry, being part of two modules as well as receiving input from module 4 and supplying information to module 1. Bilateral lesions to the HVc and AP disrupt the ability to produce new song but do not disturb learned songs (Alvarez-Buylla, 1992). These results supported a significant role for the HVc in adult learned songs.

The ability of male canaries to seasonally add and adjust components of songs, while females generally do not sing (Nottebohm, 1980), prompted Nottebohm to perform more focused analyses. In 1981 he measured the volume of the male canary HVc compared to the female HVc, in comparison to overall brain weights, and compared the results to song repertoire. He discovered that an increase in song repertoire correlated with an increase in HVc volume; furthermore, males had an increased volume of HVc and AP compared to females, with significance in excess of the sexual differences in brain weight (Nottebohm *et al.*, 1981). Intriguingly, the size of the males' testes did not correlate with song repertoire, although it did correlate with the volume of the HVc and AP. This suggested that seasonal changes in testosterone could influence the size of the high vocal centre, further supported by the HVc and AP expression of androgen receptors (Nottebohm *et al.*, 1987). In support of this, when female canaries are treated with testosterone, the volume of the HVc

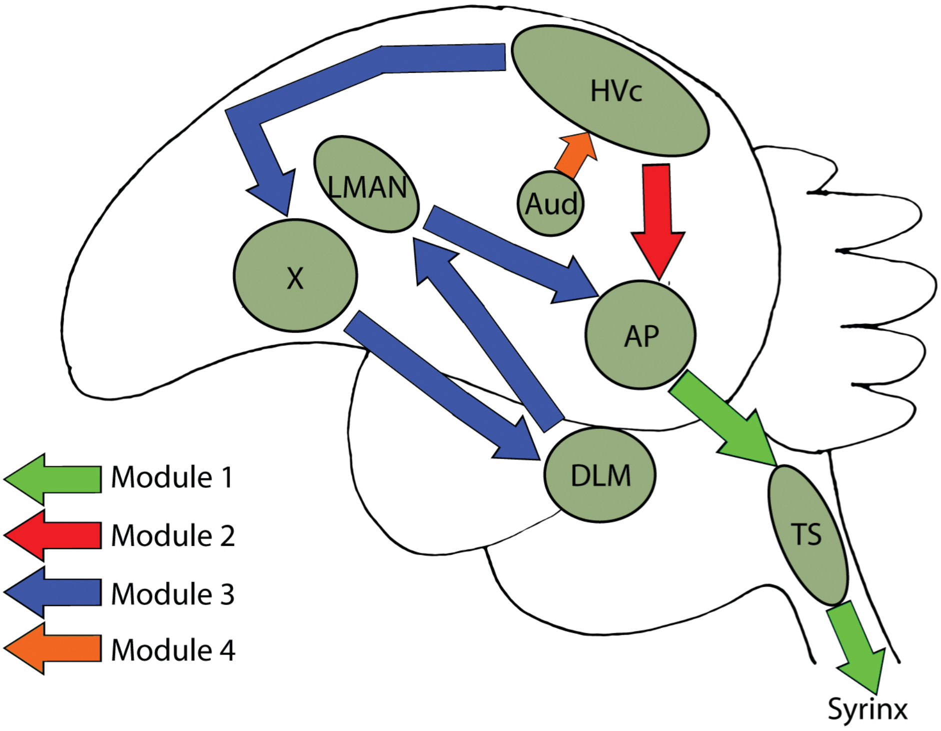


**Figure 1.1: Song circuitry**
Schematic of hierarchical song circuitry in canary brain.
TS, tracheosyringeal nerve; AP, archipallium; DLM, dorsolateral thalamus; LMAN, lateral magnocellular nucleus of anterior nidopallium; DLM, dorsolateral thalamic nucleus; X, area X of basal ganglia; HVc, High vocal centre; Aud, auditory nidopallium. The HVc is at the centre of the song circuitry: a component of modules 2 and 3, receiving input from module 4 and supplying input to module 1.

increases and they develop male-like singing characteristics (Nottebohm, 1980).

Subsequent experiments by Goldman and Nottebohm used female canaries treated with testosterone to determine whether the increase in volume of HVc in response to androgens could be attributed to adult neurogenesis (Goldman and Nottebohm, 1983). Tritiated [$^3$H] thymidine (3HT) was injected into canaries, where it was incorporated into the DNA during S-phase, labelling cells that are undergoing proliferation (Cavanagh *et al.*, 2011). During and after injection of 3HT, the birds were continuously treated with testosterone and analysed five weeks later, in comparison to controls (3HT-only females). Independent of testosterone-administration, all female canaries showed extensive 3HT labelling in the HVc and adjacent ventricular zone of the lateral ventricles (Goldman and Nottebohm, 1983). The majority of newborn cells were found to be neurons, as assessed using nissl staining or electron microscopy imaging, which revealed extensive rough endoplasmic reticulum and processes indicative of dendrites and axon hillocks (Goldman and Nottebohm, 1983; Paton *et al.*, 1985). Sacrificing the birds 48 hours after 3HT injection revealed no 3HT-positive neurons in the HVc, but large numbers of 3HT-positive glia and endothelial cells in the ventricular zone. Testosterone can increase the number of 3HT-labelled ependymal cells in the VZ overlying the HVc (Goldman and Nottebohm, 1983), and periods of neuronal addition occur as seasonal testosterone levels rise despite a neurogenic mechanism independent of testosterone (Alvarez-Buylla and Kirn, 1997; Goldman and Nottebohm, 1983), suggesting androgens increase recruitment and survival of newborn neurons (Nottebohm *et al.*, 1987; Yamamura *et al.*, 2011). Importantly, these results show that the ventricular zone of the canary lateral ventricles harbour constitutively proliferating neural progenitor cells, which generate newborn neurons that migrate into the HVc. In addition, seasonal changes in androgens alter the number of newborn neurons recruited to the HVc.

These experiments provided evidence that newborn neurons are generated in a restricted region of the adult songbird brain and are then integrated into the vocal control nuclei. Such results inferred that adult-born neurons could promote the learning and modification of song repertoire, actively remodelling networks within the HVc song nuclei (Nottebohm, 2004). Subsequent structural/morphological analyses suggested that newborn neurons integrate into active circuits, and electrophysiological recordings indicated that such integration resulted in function: they were able to respond to auditory stimuli, evoking action potentials (Goldman and Nedergaard, 1992; Alvarez-Buylla and Kirn, 1997). Half of newborn neurons were projection neurons, and the other half were interneurons (Alvarez-Buylla, 1992; Alvarez-Buylla and Kirn, 1997), supporting the notion that adult neurogenesis ensures connectivity, as well as having behavioural significance.

Interestingly, neuronal addition is inextricably linked with cell death in the HVc of the adult canary. The volume of the HVc reaches its peak within four months post-hatching; however neurons are continually generated and incorporated throughout life (Alvarez-Buylla and Kirn, 1997). Therefore there is a mechanism that ensures that cells undergo programmed cell death and that the volume of the nuclei does not continually increase with age. This relationship is heavily influenced by seasonal changes, with peak cell death in summer and winter, and peaks of neurogenesis in spring and autumn. As previously eluded to, seasonal testosterone-level fluctuations precede the recruitment of newborn neurons (Nottebohm *et al.*, 1987), while recent studies support androgenic regulation of neuronal recruitment (Yamamura *et al.*, 2011). These observations suggest the preferential loss of old neurons, perhaps to ensure the system maintains its plasticity. Indeed, when the HVc to AP projection neurons are labelled in spring, up to half of these neurons have been replaced by autumn (Alvarez-Buylla and Kirn, 1997). If the amount of old neurons in the vocal control nuclei was kept high, the stability of such networks could limit how much the circuitry may be modified and may result in an inability to adapt compared to rivals.

The adult zebra finch provides an intriguing contradiction to the results found in the canary. These songbirds do not change their song throughout life; however neurogenesis in this species leads to a net increase in the number of HVc to AP projection neurons with age (Walton *et al.*, 2012), indeed HVc to AP projection neurons are absent at birth and present in adulthood (Alvarez-Buylla, 1992). Furthermore, birth-dating studies reveal that these projection neurons can survive for at least four years (Walton *et al.*, 2012), in stark contrast to the seven-month turnover of HVc to AP projection neurons in the canary (Alvarez-Buylla and Kirn, 1997). This apparent lack of replacement in comparison to the canary supports adult neurogenesis as a mechanism that can exhibit species-specific modifications.

Unlike canaries, whose breeding pattern is dependent on seasonal changes in photoperiods, the wild zebra finch mates upon arrival of the rains in Australia and must therefore be ready to breed according to environmental conditions, which can show yearly changes (Walton *et al.*, 2012). While the relationship between neurogenesis is strongly linked to song learning and breeding in canaries, zebra finches do not produce new song; thus neurogenesis is likely linked to a different telencephalic role that increases chance of survival and breeding. It is possible to speculate that the addition of neurons to the high vocal centre in zebra finch could be associated with the individual's perception of song and its ability to recognize previous mating partners. This species can live in colonies of a thousand individuals; developing a 'phonebook' that increases with age could result in a selective advantage when the rains spontaneously arrive (Walton *et al.*, 2012). Canaries, conversely, are able to produce a new 'phonebook' and 'songbook' seasonally to increase chance of breeding.

For adult neurogenesis to be optimised to a species, a careful balance between energy cost to the individual and the chance of reproduction must be maintained. The canary and zebra finch present two opposing examples where modification to the neurogenic mechanism: birth; migration; survival, has ensured maximum efficiency and likelihood of genetic information being passed to progeny. In this case, being able to learn a variety of songs and go through periods of low energetic cost is beneficial to seasonal breeders, while having one song and continuously using energy to recognise individuals in a society benefits spontaneous breeding. This argument introduces the idea that the main influential factor for the success of adult neurogenesis is the chance of reproduction, and supports the notion that modifications to neurogenesis that can improve survival are instrumental to an individual's ontogeny and the phylogeny of a species. It follows that adult neurogenesis has a strong relationship with an organism's ability to adapt to environmental challenges, with external stressors modifying the neurogenic outcome over time, resulting in the evolutionary selection of an adaptation that provides the best chance of survival.

Though there is still much to discover about the function of adult neurogenesis in songbirds, it is clear that neurogenesis influences the replacement of neurons and remodelling of networks to adapt behaviour. Despite paradoxes between species, they continue to be an excellent model to study how the relationship between cell death and neurogenesis influences a measurable and definable behavioural outcome. Such studies have pioneered further investigation into adult neurogenesis, a feature previously thought to be limited to fish, reptiles and amphibians (Colucci-D'Amato *et al.*, 2006; Chapouton *et al.*, 2007).

### 1.1.2 Neurogenesis in the subventricular zone of the mammalian adult lateral ventricles

Many of the studies into adult mammalian neurogenesis have utilised rodent models because they provide an evolutionarily closer representation of human brain organisation than avian models. Evidence of adult neurogenesis found in mammals is more pertinent, from the perspective of biomedical science, as it provides a crucial link, tantalising clues, and potentially an understanding of function and regulation of neurogenesis that may exist in the human adult brain. Furthermore, recent advances in genetic manipulation and control of gene function have generated invaluable tools to study cell behaviour and genetic influences on a more precise scale than ever before (Dhaliwal and Lagace, 2011)(see box 1).

## Box 1.1: The Cre-lox system

A widely used genetic approach involves cre-recombinase mediated recombination of loxP sites to control the expression of genes of interest (see figure 1.2). By restricting cre-recombinase expression to specific tissues and in particular conditions, genomic sequences can be inverted, translocated and excised to allow great control over gene function (Sauer, 1987; Novak, 2000). This is particularly useful for developmental studies in adult systems, where temporal control of recombination ensures embryonic and postnatal development is not affected by gene deletion and/or an adult cell can be lineage-traced. A common technique is the use of a cre-recombinase, fused to a modified oestrogen receptor, expressed under the control of a cell/tissue-specific promoter (Mori *et al.*, 2006). The fusion protein is restricted to the cytoplasm of the specific cell type until administration of tamoxifen, a pharmacological antagonist of the oestrogen receptor, resulting in translocation to the nucleus and recombination of loxP sites. There are many modifications and alterations to this framework, but the principle has become an integral technique in the field of adult somatic stem cells. Furthermore, recent advances allow researchers to follow the fate of individual cells within an identical population based on their reporter-colour prolife, such as in the confetti and brainbow mice (Livet *et al.*, 2007; Simons and Clevers, 2011; Bonaguidi *et al.*, 2011), while conditional expression of light-responsive channelrhodopsin protein has led to the novel and invaluable ‘optogenetics’ tool, in which cells and circuitry can be experimentally activated by light (Fenno *et al.*, 2011; Sternson, 2013).

Much of our understanding of adult neurogenesis has arisen from developmental studies that analyse neurogenesis within the embryonic environment. Such studies have demonstrated that multipotent neuroepithelial cells aquire neuronal fate through the action of proneural transcription factors of the basic Helix-Loop-Helix (bHLH) class, conserved in invertebrates (Lemke, 2009). Vertebrate proneural genes, such as Neurogenins and Mash, initiate a

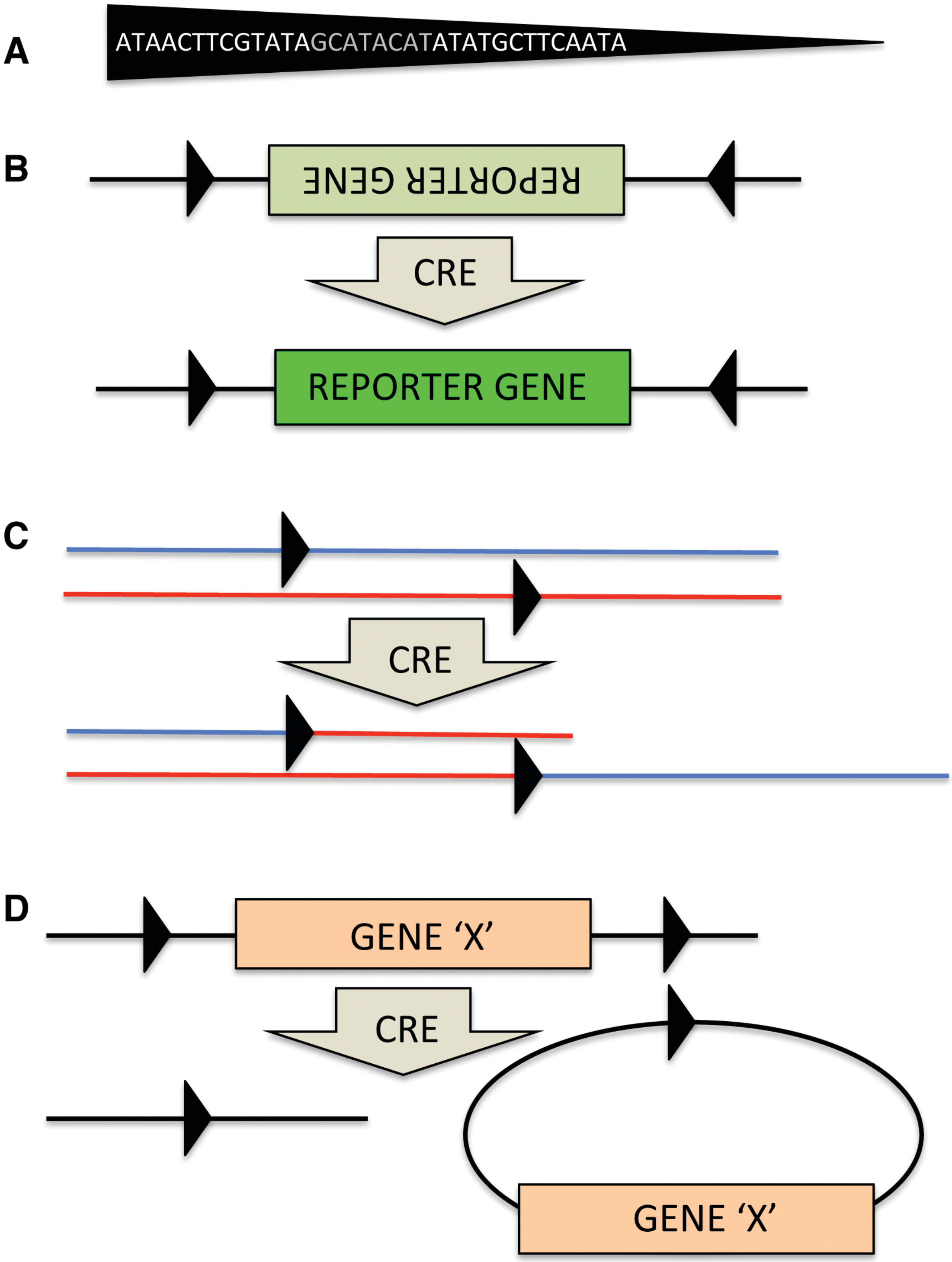


**Figure 1.2: Cre-mediated recombination of loxP sites**

A. The loxP site is a specific 34-base pair sequence consisting of a core sequence (grey) and two inverted flanking repeats (white).
B. Cre-mediated recombination of loxP sites inverts the gene of interest when loxP sites are orientated in opposite directions.
C. When loxP sites are located on different chromosomes, cre-mediated recombination leads to translocation of chromosome segments.
D. When loxP sites are located on the same chromosome in the same orientation, cre-mediate recombination excises loxP-flanked (floxed) gene.

lineage commitment to neural progenitors, where lateral inhibition, regulated by Notch and its ligand, Delta, on neighbouring cells, repress or confirm neuronal differentiation respectively (Kintner, 2002; Bertrand *et al.*, 2002). Subsequent to a robust, early generation of neurons, alternate differentiation programmes are promoted, leading to the generation of glial cells: oligodendrocytes and astrocytes. In most of the adult mammalian central nervous system, and in contrast to cold-blooded vertebrates, injury/lesions appear to stimulate this second fate, resulting in de-novo gliogenesis and the formation of glial scars, rather than in the formation of new neurons. Neurogenesis is thus not widespread in adult mammals.  However, populations of cells exist in restricted regions that retain the potential to generate all three neural cell types: neurons, astrocytes and oligodendrocytes. Similar to the situation in songbirds, the subventricular zone (SVZ) of the striatum, adjacent to the lateral ventricles, harbours such multipotent neural stem cells.

The SVZ is a classically studied region of the adult mammalian brain, and was identified as neurogenic in rodents in 1992 by Reynolds and Weiss. Studies of neurogenesis during development reveal epidermal growth-factor (EGF) to be a crucial proliferation regulator, a mitogen. The expression of EGF and its receptor in the adult striatum first suggested the exciting possibility that cells within the striatum retained neural progenitor characteristics. By dissociating cells from the EGF receptor-rich striatum, and culturing in EGF, free-floating balls of proliferating cells were observed, dubbed ‘neurospheres’ (Reynolds and Weiss, 1992). These neurospheres could be propagated and were found to differentiate into cells of the neural lineage, given the appropriate substrate. The generation of neurospheres has since been used as an in-vitro assay for neural stem cell characteristics. While routinely used, the sphere-formation method is a retrospective assay that identifies stem cells based on their self-renewal and differentiation potential when removed from their in-vivo environment. Recent studies have demonstrated that the use of mitogens can bias the true self-renewal capacity and differentiation potential of heterogenous populations of cells in vitro (Ruau *et al.*, 2008; Pastrana *et al.*, 2011; Conti and Cattaneo, 2010), studies should therefore be cautious in discerning direct conclusions of the nature of neural stem cells from neurosphere assays. Although EGF receptor-expressing cells represent a population of neural progenitors that can self-renew and differentiate in-vitro, given the limitations of the neurosphere assay, it was important to define these cells in-vivo in order to provide context to their potential.

Adult neural stem cells reside in a niche: a microenvironment generated by the stem cell that regulates its maintenance, renewal and differentiation within the wider context of the biological environment. Evidence for the importance of the SVZ niche in controlling neurogenesis is apparent from experiments that trace SVZ-derivatives, using transgenic mice that express a reporter protein downstream of a neuronal specific promoter (Lois and Alvarez-Buylla, 1994). When transgenic SVZ cells are grafted into SVZ sites in wild-type mice, reporter-positive neurons are found in the olfactory bulb, up to 8mm away, indicating long-distance migration of newborn neurons. Reporter-positive neurons are not found within the niche itself, suggesting that the local environment is not conducive to terminal neuronal differentiation. By contrast, reporter-positive neurons are observed close to graft sites when the SVZ is grafted to non-SVZ regions, presumably because the new environment does not recapitulate the conditions within the SVZ niche; there are no cues for long-distance migration and here neuronal-specified cells undergo terminal differentiation (Lois and Alvarez-Buylla, 1994). Together, these experiments indicate the importance of the environment to neural progenitors. Surrounding cells and cues can suppress or provide permissive conditions for neurogenesis and long-distance migration.

Determining the relationship of cells within the niche is vital in order to characterise the cell-types present and the identity of the neural stem cell. The SVZ niche consists of cells termed A, B, C and E cells (see figure 1.3). A-cells can be identified by immunostaining as neuroblasts: migratory cells committed to neuronal fate though not terminally differentiated, and are found in the SVZ as well as in chains along the rostral migratory stream (RMS) that connects the lateral subventricular wall with the olfactory bulb. A-cells express the immature migratory neuronal markers doublecortin (DCX) and polysialylated-neural cell adhesion molecule (PSA-NCAM). B-cells are identified by immunostaining as

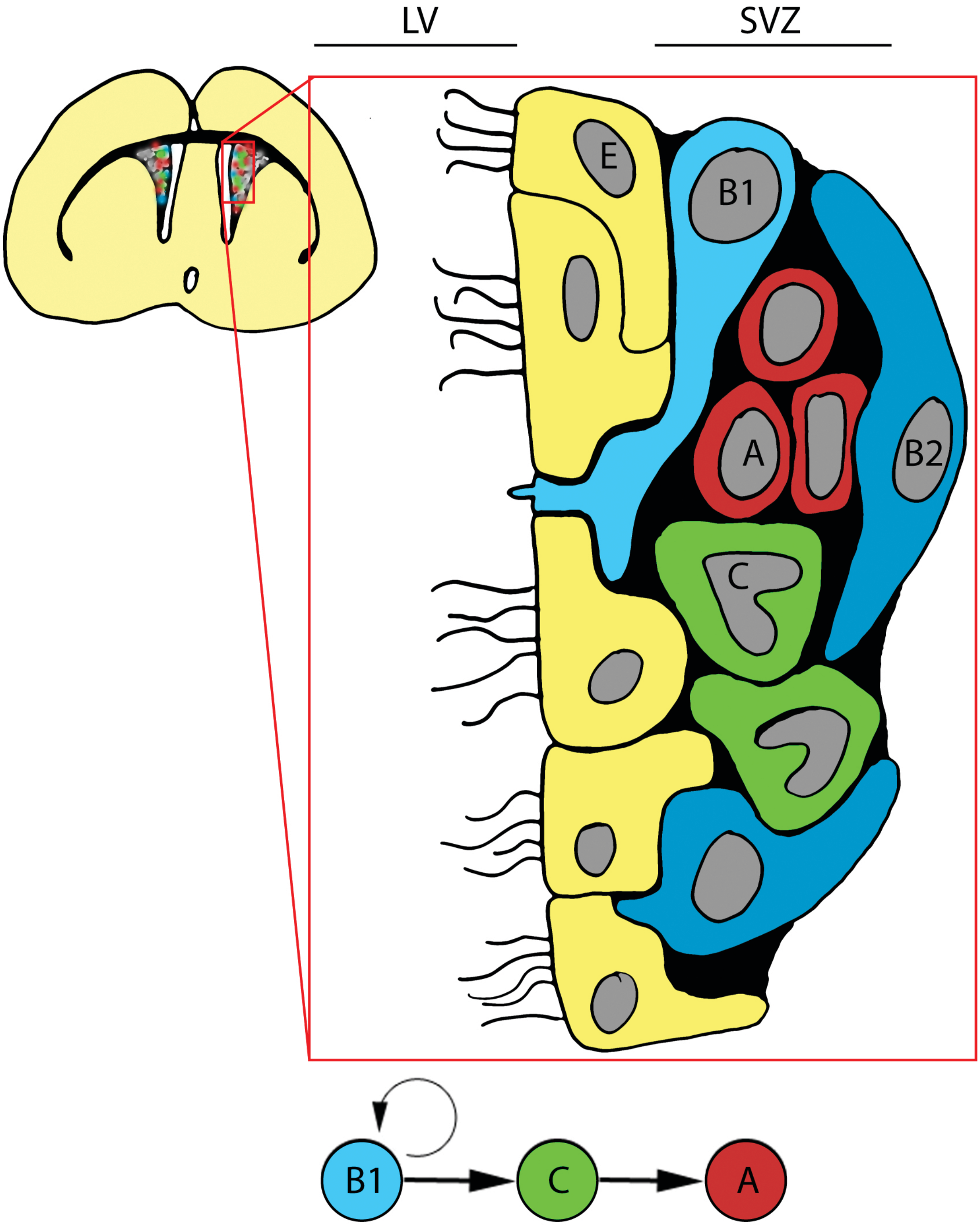


**Figure 1.3: the subventricular zone niche of the lateral ventricles**
A schematic of the SVZ is shown enlarged from a cross section of the adult mouse brain. LV, lateral ventricle; SVZ, subventricular zone; E, ependymal cell; B2, subventricular zone astrocyte. B1 cells contact the apical surface and asymmetrically divide to generate a B1 cell and a transit amplifying progenitor C cell. C cells progress down the neuronal lineage to generate A cells, immature neuroblasts.

astrocytes, expressing glial fibrillary acidic protein (Gfap), and constitute two distinct populations in the SVZ: intermediate-filament protein Nestin-positive B1-cells have a small apical surface that contacts the ventricle, while B2-cells have a stellate morphology and do not contact the ventricular surface. C-cells are a population of highly proliferative neuronal precursors that express Nestin. Lastly, E-cells are ependymal cells that line the ventricular wall and display multiple long cilia that propagate the flow of cerebrospinal fluid (CSF) through the ventricles (Doetsch *et al.*, 1999a).

As in the avian model, use of 3HT to label proliferating cells is a classic methodology to identify cells that have entered the cell cycle and retain the thymidine analogue, providing potential candidates for the slow-dividing label-retaining neural stem cell. Long-term infusion of 3HT into the lateral ventricles results in extensive incorporation in B1-cells but the absence of labelling in E-cells (Doetsch *et al.*, 1999a). Culturing ependymal cells specifically (after using an adenovirus to target the ventricle wall) does not result in the formation of neurospheres, whereas C-cells, rich in EGF receptor, generate robust numbers (Doetsch *et al.*, 1999a; Spassky *et al.*, 2005; Pastrana *et al.*, 2009). Therefore ependymal cells do not have the characteristics of a neural stem cell, whilst B1-cells retain the thymidine analogue and C-cells can generate neurospheres. Infusion of Ara-C, an anti-mitotic factor that kills proliferating cells, results in the loss of C-cells and decline in A-cells, whilst B-cells remain (Doetsch *et al.*, 1999a; Doetsch *et al.*, 1999b). 3HT infusion indicates that B1-cells go through the cell cycle as little as one hour after anti-mitotic treatment, with C-cells appearing two days later and A-cells present 5 days after treatment. Thus, B1-cells represent a population of relatively infrequently dividing cells, which are not killed by Ara-C treatment, but consequently divide and generate proliferative neural precursors and subsequent neuroblasts (Doetsch *et al.*, 1999b). Indeed, genetic labelling of B1-cells with a replication-competent reporter under Gfap promoter control, results in reporter expression in migrating neuroblasts in the SVZ and along the RMS after three days, and importantly, in granular and periglomerula neurons of the olfactory bulb after two weeks (Doetsch *et al.*, 1999a). Elegant studies using transgenic mice have shown that neuroblasts are continually generated in the SVZ, and migrate in chains through glial tubes that line the rostral migratory stream, to the granule cell and periglomerular layer of the olfactory bulb where they are destined to differentiate into interneurons (Doetsch *et al.*, 1999a; Lois and Alvarez-Buylla, 1994; Kaneko *et al.*, 2010). This experimental design reveals the identity of the neural stem cell population as the B1-cell in the SVZ, and reveals its relationship with other niche cells and the ultimate destination of its progeny (see figure 1.3). The significant questions that remain are an understanding of the factors that govern regulation of the niche, and the functional consequence of its existence.

Many neurotrophic factors and growth factors are involved in the precise control of the SVZ niche, influencing the process of proliferation, migration and neuronal differentiation. The mitogenic effect of EGF, as mentioned previously, plays a major role in regulating renewal and differentiation of stem/progenitor cells within the SVZ niche (Reynolds and Weiss, 1992). C-cells constitute the largest population of cells that express EGF receptor in the SVZ. Infusing EGF for short periods of time results in an increased number of C-cell proliferative progenitors at the expense of neuroblast A-cells: thus the thymidine analogue, bromodeoxyuridine (BrdU), is incorporated into C-cells at an increased frequency compared to vehicle-infused animals (Doetsch *et al.*, 2002). Significantly less BrdU incorporation is seen along the RMS or in the olfactory bulb, and labelled neurons are rare, in comparison to controls. Instead, BrdU-positive differentiated cells are commonly astrocytes (Kuhn *et al.*, 1997). Together, this demonstrates that increasing EGF can stimulate de-novo gliogenesis. It is evident that neurogenesis occurs through a well-regulated balance of factors: by adding weight to one side of the scales the fate of progenitors is tipped in the opposite direction. Equally, a long-term infusion of EGF causes C-cells to become highly proliferative and invasive progenitors (Doetsch *et al.*, 2002). This is of considerable interest as highly malignant glioblastoma tumours in the brain show EGF receptor gene amplification and overexpression (Lopez-Gines *et al.*, 2010), evidence of the role of EGF in self-renewal and the importance of strict regulation of self-renewal pathways.

Other important factors known to influence neural progenitor regulation are the family of fibroblast growth factors (Fgfs), notably Fgf2. During development, Fgfs are crucial for the correct proliferative, migratory and differentiation paths that pattern and organise the body (Mason, 2007). Interestingly, in the developing nervous system, Fgf2-responsive cells precede the appearance of EGF responsive cells (Doetsch *et al.*, 2002). Further investigation is required to discern whether this developmental timing confers differences between true neural stem cells and more committed progenitors. In the adult SVZ, Fgf2 is expressed by B-cells and the receptors FgfR1 and FgfR2 are expressed on C-cells (Frinchi *et al.*, 2008). Infusion of FGF2 increases the numbers of C-cells that incorporate BrdU, like EGF, but in contrast, after FGF2 infusion has ceased a large number of newborn BrdU-positive cells are released into the RMS and go on to generate olfactory bulb neurons (Kuhn *et al.*, 1997). Thus, FGF2 has strong mitotic influences in the SVZ, but also supports and permits neurogenesis.

Another signalling pathway that is of great significance in SVZ neurogenic regulation is that of the Notch-signalling cascade. Notch is a single-pass transmembrane receptor, which, through binding of its ligands present on adjacent cells, controls the maintenance of neural progenitors in the embryonic neural tube. Aberrant activation of the Notch pathway during development results in the expansion of the neural progenitor population at the expense of the generation of newborn neurons (Gaiano *et al.*, 2000; Basak and Taylor, 2007), similar to the effects seen from EGF infusion in the adult SVZ (Doetsch *et al.*, 2002). Both Notch1 and its ligand, Jagged1, are expressed in the adult SVZ niche and show a complementary expression pattern, with Jagged1 present on Gfap-positive subependymal cells and Notch1 expressed on Nestin-positive B1-cells (Nyfeler *et al.*, 2005). Homozygous mutants for either *Notch1* or *Jagged1* are embryonic lethal, however a double-heterozygous mutant for both is viable but shows a significant decrease in proliferative cells in the SVZ and RMS compared to wildtype mice, supporting Notch-signalling as an important point of control for regulating division and differentiation. In support of this, Jagged1 can stimulate proliferation of neurospheres in culture at a comparable level to EGF; however Jagged1 increases the proportion of neurons that differentiate and Notch1 can increase the proportion of differentiating glia in-vitro (Nyfeler *et al.*, 2005). These data support a reciprocal relationship between cells expressing ligands and receptors in a precisely controlled balance that dictates the rate of renewal as well as the fate of progeny. The expression profiles of ligands and receptors suggest that these factors exert their effects at the level of neural stem and progenitor cells to influence differentiation, rather than exerting an influence further downstream.

Sonic hedgehog (Shh) is a further signalling protein that has been identified to have a significant role during development in patterning of the nervous system, and indeed, is implicated in the control of the adult SVZ niche. Studies show that *Shh* expression is conserved into adulthood in murine SVZ cells. Additionally, components of the Shh signalling pathway are expressed differentially by niche cells, with purified populations of B- and C-cells being particularly rich in expression of the Shh effectors, *Gli1* and *Gli2*, as well as the Shh receptor (*Ptc*), while expression is low or absent in A- and E- cells (Palma *et al.*, 2005). Evidence for the role of Shh in the SVZ niche is provided by injection of its inhibitor, cyclopamine, resulting in a decrease in BrdU incorporation compared to control mice. Furthermore, after 30 days there is a marked reduction in the number of newborn neurons in the olfactory bulb (Palma *et al.*, 2005), supporting a role for Shh in regulation of proliferation and neurogenesis. Like other factors described above, Shh exerts its neurogenic effects at the level of the neural stem cell and not further downstream, for example in migration or survival of newborn neurons. A separate study that used Gli1::CreERT2 transgenic mouse line to identify and genetically fate map Shh responsive cells revealed reporter-positive cells in the SVZ, and an absence in the RMS and olfactory bulb after acute sacrifice (Ahn and Joyner, 2005). However, after a long-term chase, reporter-positive progeny of Shh responsive cells could be detected in the migratory stream and in the olfactory bulb, and were increased in number within the SVZ suggesting expansion of the original genetically-labelled cells (Ahn and Joyner, 2005). Taken together, factors that govern neurogenesis during development, including EGF, Fgfs, Notch, and Shh, have conserved and important roles in regulating adult neurogenesis within the SVZ niche (summarised in table 1.1).

**Table 1.1: Summary of the influence of factors upon the adult SVZ niche**

|  | EGF<br>**Doetsch - 2002**<br>**Kuhn - 1997** | FGF<br>**Kuhn - 1997** | Notch<br>**Nyfeler - 2005** | Shh<br>**Palma - 2005** |
|---|---|---|---|---|
| Proliferation | **+** | **+** | **+** | **+** |
| Neurogenesis | **-** | **+** | **-** | **+** |
| Summary | Maintain progenitor pool | Regulate neurogenesis | Maintain uncommitted progenitors | Regulate neurogenesis |

Table 1.1 summarises the influence of neurodevelopment factors on the adult SVZ niche, in the context of progenitor proliferation and neurogenesis. An increase is represented by '+', while a decrease is represented by '-'.

Neural stem and progenitor cells are in a landscape that is being continuously remodelled, and evidence is emerging to suggest that the surrounding cells/ signalling molecules regulate proliferation and commitment of cells appropriate to context. For instance, the SVZ can decrease production of neurons and switch to an increase in oligodendrocyte production in response to the loss of myelin sheaths, which support propagation of action potentials along neuronal axons. Gfap-expressing B1-cells give rise to a small proportion of Olig2-positive oligodendrocyte precursors in-vivo. Olig2 is a transcription factor, controlled by Shh, that binds to DNA and inhibits transcription of proneural genes: thus, dysfunction in Olig2 (using a retroviral vector containing a dominant-negative construct) diverts precursors to a neuronal fate (Ninkovic and Gotz, 2013). However, in the presence of bone morphogenetic protein (BMP), Olig2 is sequestered and proneural genes can be transcribed, although BMP itself stimulates astroglial genesis at the expense of neurons. Normally in the SVZ niche, a tightly regulated balance of BMP and inhibitors (such as Noggin) are found in order to direct neuronal or astrocyte differentiation and limit oligodendrocyte precursors (Lim *et al.*, 2000). However, in response to demyelination, the process by which neurons lose their oligodendrocyte insulation, BMP inhibitors are released and Olig2 causes a massive downregulation of proneural genes, and a subsequent increase in oligodendrocyte-fated cells to compensate for the loss (Ninkovic and Gotz, 2013). The intricate relationship between the myriad of niche signalling factors is inherently complex, however the balance is regulated in order to maximise the context-dependent differentiation whilst ensuring strict control on proliferation.

From the example above, it is apparent that Shh and BMPs have a complementary role on the niche in controlling differentiation. Interestingly, these antagonistic functions are reminiscent of their actions during development, and support a conserved function in the adult neurogenic niche. During embryogenesis, *Shh* is expressed in the ventral midline of the newly formed neural tube, the floor plate, induced by the Shh from the underlying notochord (Wilson and Maden, 2005). Shh is secreted from these two ventral sources, generating a concentration gradient along the dorsoventral axis of the neural tube. The level of Shh that neural progenitors in the ventricular zone of the neural tube receive is dependent upon the distance from the source (Briscoe *et al.*, 2001; Altaba *et al.*, 2003; Wilson and Maden, 2005), and importantly, the differences in concentration directs the patterning of neurogenesis by conferring positional information upon progenitors (Dessaud *et al.*, 2008; Wolpert *et al.*, 2002). Concurrently, notochord explants placed adjacent to the dorsal neural tube result in an ectopic floor plate and ventralisation of this region (Placzek, 1995). Shh therefore acts as morphogen to direct proliferation and differentiation of progenitors into neuronal subtypes, such as motorneurons, by activation of the Gli transcription factors. This is supported by studies that electroporate a mutant Shh receptor, Patched (mPtc$^{loop2}$), in the developing chick neural tube (Briscoe *et al.*, 2001). Shh cannot bind to the mutant Ptc, and so does not exert its long range signalling effects, resulting in a dorsalisation of the neural tube. In contrast to Shh, BMPs are secreted from the dorsal roof plate, creating a secondary signalling centre in the neural tube that antagonises the ventral signal (Lemke, 2009). The concentration of both Shh and BMPs dictate the exact identity of neuronal populations by their relative suppression and activation of transcription factors along the dorsoventral axis. Of particular relevance, regions of *Shh* and *Bmp* expression are maintained in discrete locations of the adult nervous system, including the SVZ (Palma *et al.*, 2005; Lim *et al.*, 2000), where their role in patterning neurogenesis is strikingly similar to the developmental program.

Under control conditions in the adult SVZ, the signalling pathways detailed above ensure the continuous addition of neurons to the olfactory bulb; however in experimental and disease conditions the delicate equilibrium provided by the niche is exposed to pressures that can result in inappropriate proliferation or aberrant differentiation. The significance of an imbalance in factors or the dysfunctional regulation of the niche can be contextualised by considering the physiological importance of SVZ neurogenesis. In rodents, over 30,000 newborn neurons reach the olfactory bulb every day (Nissant *et al.*, 2009), the area of the nervous system that is responsible for sensory smell information processing. This has an intriguing functional significance, as the addition of neurons to mature circuits suggests that SVZ neurogenesis has an impact on the plasticity of adult networks. As migratory neurons from the SVZ require NCAM for their migration and subsequent incorporation into the olfactory bulb, the generation of *NCAM* mutant mice provides an opportunity to uncover the detriment to the organism when cells fated for interneurons do not reach their destination. Deletion of the NCAM gene does not prevent the mouse from recognising odorants to which it has been recently exposed (less than 60 minutes) (Gheusi *et al.*, 2000). However, *NCAM* mutants spend significantly less time investigating novel odours compared to wildtype mice; further, mutant mice show no difference in time spent investigating novel odours compared to reinforced odours, whereas wildtypes show a marked difference (Gheusi *et al.*, 2000). These results suggest that a lack of newborn interneurons confers a reduced ability to distinguish between new odours and supports the notion that de-novo generated inhibitory interneurons in the granule cell layer are important in the refinement of olfactory discrimination. The continuous remodelling of the granule cell layer, with newborn neurons replacing older neurons committed to cell death, can be considered a necessity to increase the likelihood of survival and reproduction.

The ability to recognise new odours compared to old odours, and the subsequent learning and memory association is of great importance in differentiating between nourishment and toxins; new and previous mating partners; allies and rivals; offspring and predators. As in the songbird, adult neurogenesis presents an opportunity to manipulate networks dependent on the changing environment in order to maximise adaptation.

Electrophysiological recordings made on newborn olfactory interneurons, compared to mature counterparts, reveal a significant difference in their synaptic plasticity. Immature granule neurons, derived from the SVZ, transiently show long-term potentiation (LTP) (Nissant *et al.*, 2009), an enhancement in signal transmission between neurons. LTP is considered vital to synaptic plasticity, and is a major mechanism underlying learning and memory. The ability of immature neurons to form dynamic synapses that can increase in strength, (unlike mature granule neurons) demonstrates the importance of synaptic plasticity in odour discrimination, as well as the importance of neurogenesis to provide a modifiable network. Therefore, an important function of adult SVZ neurogenesis is to ensure strong synaptic connections are associated with novel odours, that can be refined according to experience.

The investigation into SVZ neurogenesis has extended into humans in order to determine whether the neural stem cell hallmarks observed in rodents, and other primates, are conserved. Observations made of the human lateral ventricles in neonates show an anatomically similar SVZ niche, comprising the cell types found in low-order mammals, as well as a rostral migratory stream where immature neurons can be found (Sanai *et al.*, 2004). After 6 months of age, the number of EGFR-positive cells declines, and the SVZ takes on an organisation unique to humans. Separated from the ventricular wall by a gap, a band of cells consisting of astrocytes can be found, termed the ‘astrocyte ribbon’ (Sanai *et al.*, 2004). Proliferative markers are expressed by astrocytes within the ribbon, and astrocytes can be stimulated to undergo proliferation ex-vivo. Thus, it is possible to generate robust numbers of multipotent neurospheres from this region, indicating that neural stem cells reside in the SVZ of the adult human brain (Sanai *et al.*, 2004). An additional difference observed in humans is a lack of chain migration of immature neurons through the RMS, although individual and pairs of PSA-NCAM-positive immature neurons are present (Sanai *et al.*, 2004). In accordance, post-mortem analysis of the brains of patients that have been administered a thymidine analogue clinically, reveals BrdU incorporation in mature neurons, suggesting newborn neurons may reach the human olfactory bulb in the absence of chain migration (Curtis *et al.*, 2007).

Despite the presence of neural progenitors in the adult, it is apparent that SVZ neurogenesis rapidly declines during childhood and adolescence, perhaps due to a gradual decline in requirement for synaptic plasticity at the olfactory bulb after sexual maturity and as individuals get older. Additionally, while neurogenesis is observed to decrease over the lifetime of humans, marked differences exist in the extent of this reduction between many studies of human autopsied tissue (Curtis *et al.*, 2007; Sanai *et al.*, 2004). These results prevent a definitive understanding of the age-related decline in human neurogenesis and could be the consequence of genetic variability between individuals as well as the experiences they have encountered during life, both physiologically and pathologically.

I consider the best examples of the importance of the SVZ neurogenesis in the adult human to be emerging from detailed studies of pathological conditions, such as Parkinson's Disease (PD). PD is characterised by a loss of dopaminergic neurons, most notably those of the substantia nigra that are required for motor control, leading to major symptoms such as tremors, akinesia and bradykinesia. Intriguingly, one of the first symptoms to occur in PD patients is the inability to discriminate odours (Hoglinger *et al.*, 2004)**,** similar to *NCAM* mutants (Gheusi *et al.*, 2000) (section 1.1.2, page 16). This characteristic suggests new neurons are recruited to the olfactory bulb in healthy individuals but not in PD patients. A study addressing this in mice observes dopaminergic neurons innervating EGFR-positive cells within the SVZ in mice, and finds the expression of dopamine receptor D2L on C-cells (Hoglinger *et al.*, 2004), supporting a role for dopamine in the SVZ niche. Injecting the toxic dopamine analogue (6-hydroxydopamine) to selectively kill dopaminergic neurons, a model for PD, results in a significant decrease in proliferating cells within the SVZ compared to controls; as well as a reduction in the number of cells in the

RMS and olfactory bulb that have incorporated BrdU. The use of physiological dopamine precursor, levodopa, rescued this effect (Hoglinger *et al.*, 2004), supporting dopamine as a regulator of neurogenesis. Concomitantly, human patients with PD were found to have fewer proliferative cells and immature neurons in the RMS compared to control brain tissue (Hoglinger *et al.*, 2004), giving evidence that loss of dopaminergic neurons that innervate the SVZ causes dysregulation of adult neurogenesis. While loss of olfactory discrimination is an established early symptom, future research could identify further consequences of decreased numbers of adult-born neurons in the aetiology of PD, and may identify the SVZ niche as a direct therapeutic target.

In summary, although identified only 20 years ago, great strides have been made in our understanding of mammalian adult neurogenesis. Early studies provided a rapid in-vitro assay for self-renewal and neural differentiation that is now widely used alongside thymidine analogues, marker expression and genetic engineering strategies to interrogate the SVZ niche. The niche is crucial in governing the regulation of divisions and commitment of progeny to the different neural lineages, as well as in maintaining a precise balance of signalling factors required to preserve control over neurogenesis. The signalling pathways that regulate and pattern the development of the embryonic nervous system are maintained in the adult SVZ niche, where they act upon neural progenitors in a similar manner. The presence of a neural stem cell in the SVZ is conserved in all mammals studied, predominantly functioning to ensure a robust plasticity of neural networks responsible for olfactory discrimination. The human SVZ niche has been identified, and species-specific modifications can be found, possibly due to the differential requirements of neurogenesis dictated by the external environment. Future research is likely to distinguish the contribution that SVZ neurogenesis has made to human evolution and cognition, as well as identify new roles in the natural history of neurological diseases.

### 1.1.3. Neural stem cells in the subgranular zone of the adult dentate gyrus

A further well-defined neurogenic niche also existing within the mammalian brain is located within the dentate gyrus of the hippocampus. The hippocampus or ‘hippocampal formation’ is comprised of granule neurons in the region of the dentate gyrus, followed in a curve by pyramidal neurons of the Cornu Ammonis regions (CA). CA4, or the hilar region, underlies the dentate gyrus. Between the hilar region and the granule cell layer of the dentate gyrus, the subgranular zone (SGZ) exhibits proliferation and neurogenesis in many mammals studied (Amrein *et al.*, 2011), including humans (Knoth *et al.*, 2010). An interesting difference when considering the full process of neurogenesis, as defined by proliferation, fate determination and survival, is the relatively small size of the SGZ niche compared to the SVZ. In the SVZ, neurogenesis occurs in a broad region along the entire anterior-posterior extent of the lateral ventricles (Mirzadeh *et al.*, 2008), and newborn neurons are destined to populate a distant site, the olfactory bulb. By contrast, the process of proliferation, differentiation and integration from SGZ progenitors all occurs within the dentate gyrus, as newborn neurons integrate into the adjacent granule cell layer (GCL) (Kuhn *et al.*, 1996; Gage *et al.*, 1998). This makes the dentate gyrus niche an attractive model for broadening our understanding of mammalian neurogenesis. Further, the hippocampus has crucial functions in consolidating short-term memories into long-term memories, which is important for an organism’s ability to spatially navigate its environment and to learn from experience, to increase the chance of survival. Such characteristics of hippocampal neural circuits make SGZ neurogenesis incredibly pertinent to human health and disease.

As in the SVZ, substantial evidence for a multipotent neural stem cell population within the SGZ accumulated around the turn of the 21st century. Initial studies generated neurospheres from the hippocampus, and by passaging the neurospheres several times, researchers were able to generate free-floating balls consisting of progenitors alone, which could then be dissociated and marked with a reporter gene, using retroviral infection methods (Palmer *et al.*, 1997). Single reporter-positive progenitor cells could then be expanded to generate clones, which, upon removal of growth factors, differentiated into the three neural lineages, expressing markers for neurons, glia and oligodendrocytes (Palmer *et al.*, 1997). Such studies provide evidence that cells within the hippocampus are able to self-renew through multiple passages and are multipotent in-vitro. However, as discussed already, in-vitro conditions can place artificial bias on the system of study, for example by changing the epigenetic landscape of lineage-restricted progenitors to a more undetermined characteristic (Enver *et al.*, 2005). As already stated, it is important to perform parallel studies on a neural stem cell population in-vivo in order to characterise its true potential, albeit that this presents a challenge in defining the neural stem cell within its niche.

As discussed above, a classical methodology for identifying neurogenesis and neurogenic stem/progenitor cells in the adult is the use of thymidine analogues (Cavanagh *et al.*, 2011): incorporation of 3HT into DNA provides a read-out of proliferation, while the co-expression of neuronal markers provides an indication of neurogenesis. Intraperitoneal injection of BrdU in adult mice, followed by a brief two-hour chase, reveals that the majority of proliferative cells within the SGZ co-express Gfap, a classic astrocytic marker that is also expressed by SVZ B-cells (Seri *et al.*, 2001). After a 24-hour chase period, the proportion of Gfap-positive cells that incorporate BrdU decreases, suggesting that this population either dies or no longer expresses Gfap. Such short-term analysis reveals two distinct types of proliferative cells within the SGZ: cells that are ultrastructurally similar to SVZ B-cells, as well as a Gfap-negative population (Seri *et al.*, 2001). Anti-mitotic treatment with Ara-C destroys proliferative cells, and can therefore be used to determine if there is a lineage relationship between these two mitotic populations. After Ara-C treatment, the number of BrdU-positive cells significantly decreases, followed by a significant increase and a recovery to wild-type levels two weeks later (Seri *et al.*, 2001). Cells that survive the antimitotic treatment are B-cell-like Gfap-positive cells; four days later BrdU-positive, dark Gfap-negative cells appear, and eventually BrdU-positive neurons can be found. This suggests that Gfap-positive cells are a neural stem cell population within the SGZ that can survive anti-mitotic treatment, and can proliferate to generate neurons through an intermediate Gfap-negative cell type (see figure 1.4). Subsequent experiments, using a transgenic mouse to mark Gfap-positive cells and their progeny confirmed this conclusion (Seri *et al.*, 2001). Studies have also further characterised the populations of cells within the SGZ, showing that the Gfap-positive neural stem cell expresses the embryonic neural progenitor markers Sox2, Nestin and Brain lipid binding-protein (BLBP), while the hippocampal intermediate neuronal progenitors express doublecortin (DCX) (Kriegstein and Alvarez-Buylla, 2009). These results show striking similarity between the SGZ and the SVZ niches; it should be noted, however, that this does not provide evidence that the SGZ niche is regulated by the same factors that regulate the SVZ niche (summarised in table 1.1/1.2).

Similar to SVZ-derived cells, epidermal growth factor (EGF) and basic fibroblast growth factor (Fgf2) are both required for the in-vitro culture of SGZ neural stem and progenitor cells. However, while EGF infusion results in increased proliferation and de novo gliogenesis in both the SVZ and SGZ, infusion of FGF2 increases proliferation only in the SVZ; no effect of FGF2 on proliferation or neurogenesis in the SGZ is observed upon its infusion (Kuhn *et al.*, 1997). These observations support the idea that the two niches are both broadly regulated by EGF, but suggest marked difference in their responsiveness to FGF2. Such differences in regulation are likely to reflect the different ontogeny of each niche, and the different factors to which they are exposed during their development and maintenance. Although, as yet, we do not fully understand these potential differences, a picture is emerging of the different roles of signalling factors within the SGZ niche, compared to the SVZ niche

As outlined in section 1.1.2, *Shh* is known to be crucial in the patterning of the embryonic nervous system, and has been shown to be involved in the regulation of the SVZ niche (see page 20 above). The Gli1::CreER$^{T2}$ transgenic line identifies Shh responsive cells in both the early postnatal SVZ and SGZ (Li *et al.*, 2013), as well as the adult niches (Encinas *et al.*, 2011; Ahn and Joyner, 2005), supporting an important and maintained role for *Shh* during development of the niche. Conditional mutation of *Shh*, or its downstream signalling factor,

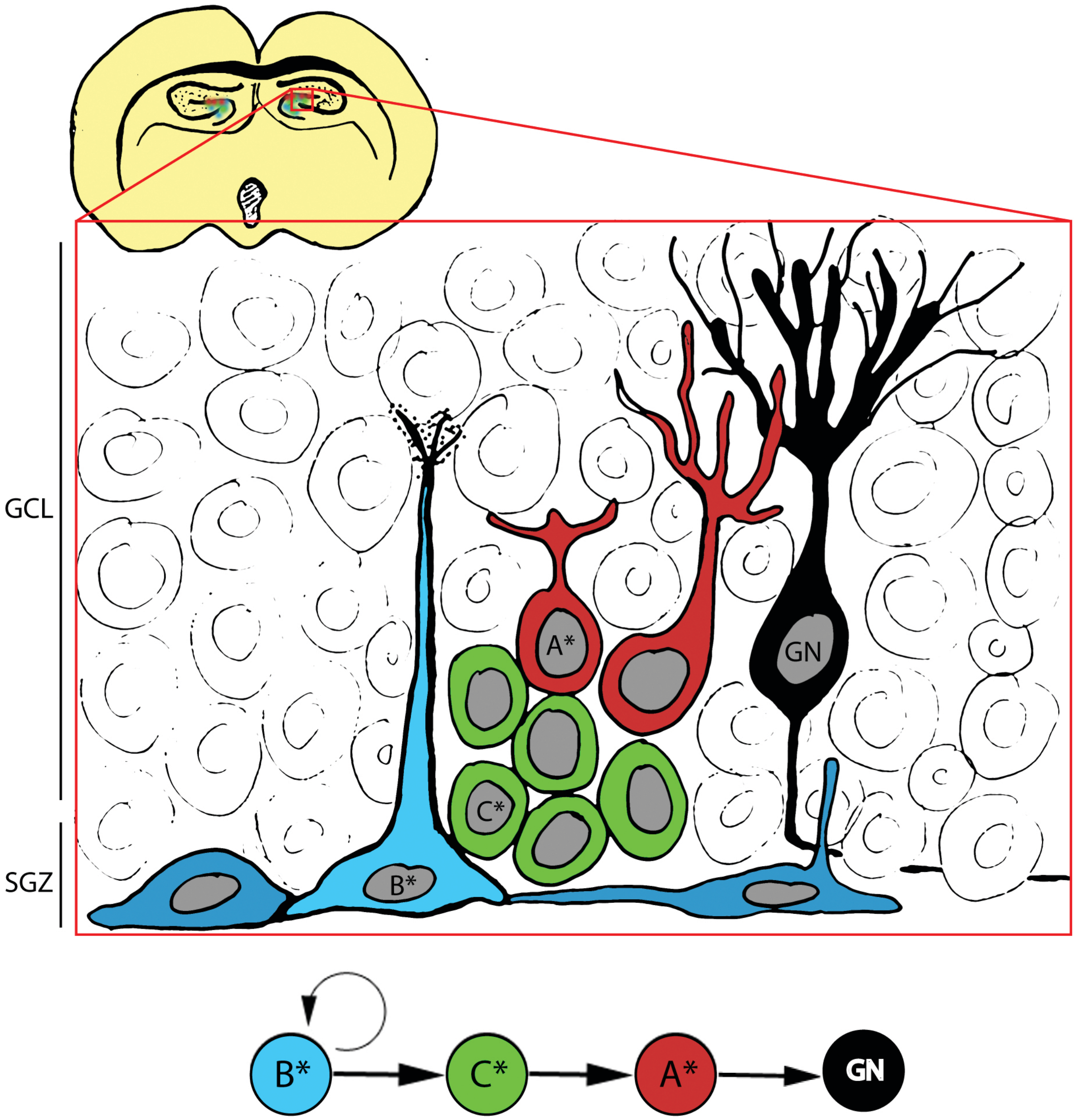


**Figure 1.4: the subgranular zone niche of the dentate gyrus**
A schematic of the SGZ is shown, enlarged from a cross section of the adult mouse brain. SGZ, subgranular zone; GCL, granule cell layer. GFAP-positive B1-like (B*) cells asymmetrically divide to generate a B1-like cell and a GFAP-negative intermediate progenitor (C*) cell. C* cells progress down the neuronal lineage to generate immature neuroblasts (A*). Immature neuroblasts mature into granule neurons (GN) in the GCL.

Smoothened (*Smo*) leads to a severe hippocampal phenotype, that includes absence of a dentate gyrus, absence of Gfap-positive neural stem cells and a failure to incorporate BrdU (Han *et al.*, 2008). This phenotype matches that of a transgenic model in which *Sox2* is deleted in Nestin-positive neural stem cells. In wild-type mice, Nestin, Sox2 and Shh are expressed together, and indeed the *Sox2*-deleted model leads to a lack of Shh in Nestin-positive SGZ neural stem cells, accompanied by a significant decrease in neurogenesis (Favaro *et al.*, 2009). This suggests that *Sox2*-dependent Shh regulation is crucial for the development and maintenance of hippocampal neural stem cells. In support of this, chromatin immunoprecipitation reveals *Shh* as a direct target of *Sox2* in the embryonic brain, while pharmacological activation of the sonic-hedgehog pathway can partially rescue the absent proliferation and massive cell death phenotype observed in *Sox2*-deficient mice (Favaro *et al.*, 2009). These experiments suggest a model in which the SGZ niche is under regulation of Shh at the level of the neural stem cell. The model suggests that *Shh* acts to preserve the stem cell pool and prevent cell death, allowing subsequent factors to control neurogenesis.

Further signalling molecules that play a crucial role during development of the nervous system, and that have been shown to have regulatory roles in the adult SGZ are the bone morphogenetic proteins (BMPs) (Lim *et al.*, 2000). BMP receptor-1A (BMPR-1A) is expressed on SGZ neural stem cells, while BMPR-1B is expressed on newborn neurons, supporting the notion that BMPs in the niche play divergent roles, elicited through signalling via the different receptors (Mira *et al.*, 2010). Deletion of BMPR-1A results in an initial increase in proliferation; however 28 days later a significant reduction of proliferation is observed compared to controls, together with a decrease in Sox2-positive cells and DCX-positive cells (Mira *et al.*, 2010). Combined with in-vitro data, Mira *et al.* (2010) demonstrate that signalling through BMPR-1A is required to maintain the pool of SGZ neural stem cells, the absence of this receptor resulting in increased proliferation and consequent exhaustion of the neural stem cell pool. During development, Shh and BMPs have opposing morphogenic effects that together are crucial for the correct patterning of the nervous system (Placzek, 1995; Lemke, 2009). Much like their effect on progenitors within the embryonic nervous system, it appears that both Shh and BMPs maintain the adult neural stem cell, but while loss of Shh results in decreased proliferation as well as increased cell death, loss of BMP results in increased proliferation and exhaustion of the pool of neural stem cells. Thus, factors that regulate embryonic progenitor proliferation and fate may operate in a mechanistically similar fashion to regulate adult neural stem cells. From these two examples alone, it is clear that the SGZ niche is maintained in a delicate balance between survival and exhaustion. Factors that provide further control on the fate of these neural stem cells are thus fundamental to the success of the niche and its downstream physiological roles.

One such crucial regulator is Notch signalling. Notch signalling drives neurogenesis in the embryonic neural tube through the process of lateral inhibition, as Notch-expressing progenitors remain uncommitted and ligand-expressing cells undergo neuronal differentiation (Lemke, 2009). Much like its importance in SVZ neurogenesis (section 1.1.2, page 19), Notch is vital for the correct neurogenic process in the SGZ. However the regional effects of Notch on SVZ and SGZ neurogenesis are seemingly contrary to each other. While double heterozygous mutants of *Notch1* and *Jagged1* result in decreased proliferation in the SVZ (Nyfeler *et al.*, 2005), conditional knock-out of the canonical Notch signalling effector *RBPjk* results in increased proliferation of Sox2-positive cells in the SGZ (Ehm *et al.*, 2010). Upon activation of Notch receptor by one of its ligands, Delta-like or Jagged, the Notch intracellular domain (NICD) is cleaved and translocated to the nucleus where it turns the transcriptional repressor complex RBPjk into an activator of target transcription factors such as the Hes family (Basak and Taylor, 2007). Hence, in the RBPjk conditional knock-out mice, the canonical Notch signalling pathway cannot be completed and Hes family members are not activated. In these mice, an initial increase in proliferation and neurogenesis is observed, and as in BMPR-1A deficient mice, this is followed by a loss of Sox2-positive cells and DCX-positive newborn neurons: an exhaustion of the proliferative pool (Ehm *et al.*, 2010). Such results suggest that canonical Notch signalling can influence BMP signalling. Interestingly, NICD can increase the expression of Sox2 in culture, while dominant negative RBPjk inhibits this (Ehm *et al.*, 2010), supporting a role for canonical Notch signalling in Sox regulation, and thus in the regulation of Shh (section 1.1.3, page 31). Notch signalling therefore presents a central mechanism to control an equilibrium between quiescence and neurogenesis, with fluctuations in activity regulating the fate of the SGZ neural stem cell. The apparent opposite effects of Notch on proliferation in the SVZ may be attributed to non-canonical Notch signalling, offering a further point of regulation on neurogenesis.

**Table 1.2: Summary of the influence of factors upon the adult SGZ niche**

| | EGF<br>**Kuhn -1997** | FGF<br>**Kuhn - 1997** | Shh<br>**Han – 2008**<br>**Favaro – 2009**<br>**Ahn - 2005** | BMP<br>**Lim – 2000**<br>**Mira - 2010** | Notch<br>**Ehm - 2010** |
|---|---|---|---|---|---|
| Proliferation | **+** | No effect | **+** | **-** | **-** |
| Neurogenesis | **-** | No effect | **+** | **-** | **-** |
| Summary | Maintain progenitor pool | Further investigation required | Maintain progenitor pool/ regulate neurogenesis | Maintain uncommitted progenitors | Maintain uncommitted progenitors |

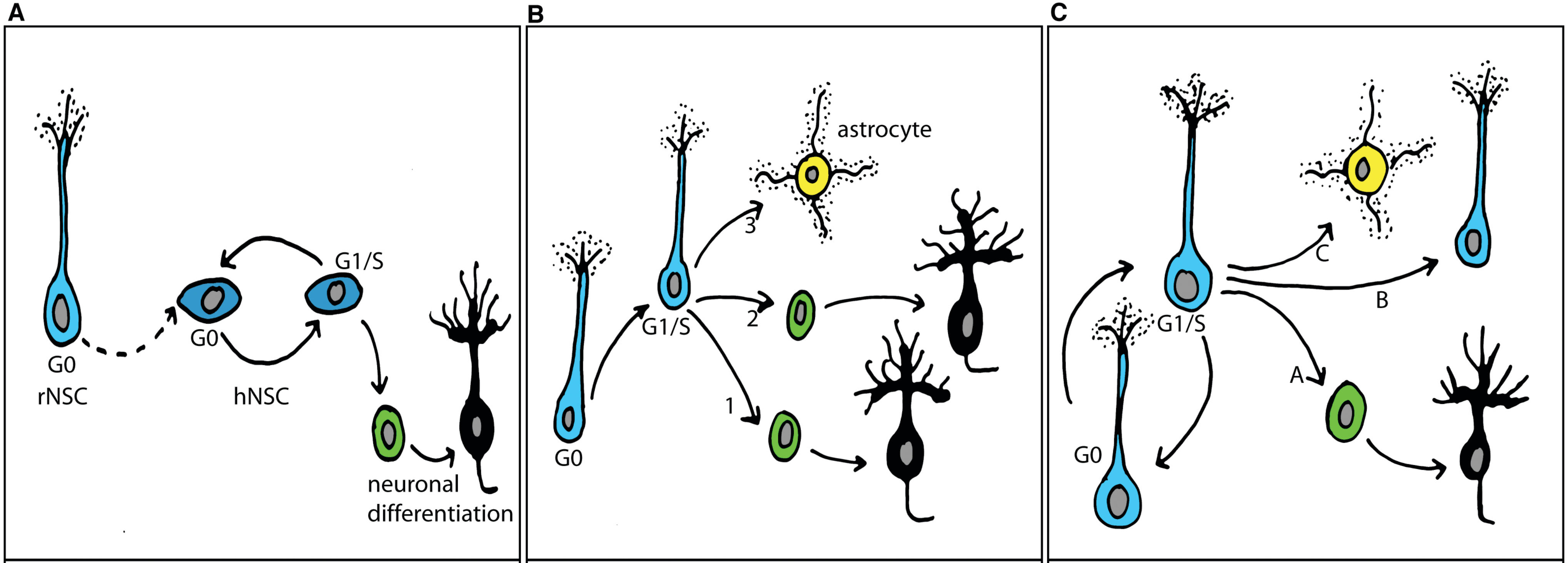
Table 1.2 summarises the influence of neurodevelopment factors on the adult SGZ niche, in the context of progenitor proliferation and neurogenesis. An increase is represented by '+', while a decrease is represented by '-'.

The central role of Notch in neural stem cell populations means that Notch/Notch signalling elements can be harnessed as useful tools to characterise progenitor populations within the adult brain. Transgenic mice that express a GFP-reporter fused with Hes5, a target of canonical Notch signalling, can be used to identify neural stem cells in which Notch signalling is activated. Hes5::GFP-positive cells can generate neurospheres in-vitro, while negative cells cannot, supporting Hes5 as an indicator of cells with neural stem cell characteristics (Lugert *et al.*, 2010). Studies using such models, however, have revealed a striking amount of heterogeneity in progenitor populations, prompting a reassessment of the traditional view that a homogenous population of neural stem cells divide asymmetrically to give rise to intermediate progenitors that go on to differentiate into granule neurons. In-vivo, Hes5::GFP-positive reporter-positive cells identify three different neural progenitor populations; quiescent radial cells, quiescent horizontal cells and proliferative horizontal cells (Lugert *et al.*, 2010). Double-thymidine analogue incorporation reveals that reporter-positive cells can enter, exit and subsequently re-enter the cell cycle, a defining feature of adult neural stem cells. Furthermore, quiescent Hes5::GFP-positive cells survive Ara-C treatment (Lugert *et al.*, 2010). Interestingly, however, it is the horizontal population that then proliferates and regenerates the lost niche cells, while the radial population remains quiescent, leading to the suggestion that radial cells provide a reserve pool of neural stem cells (Lugert *et al.*, 2010). Other studies, however, using a Nestin::Cre reporter mouse, report contradictory results, documenting evidence that the number of radial quiescent neural stem cells decreases with age but coincides with an increase in reporter-positive cells with stellate morphology and Gfap expression (Encinas *et al.*, 2011). This led investigators to suggest the 'disposable stem cell' model (Encinas *et al.*, 2011), in which radial cells have limited renewal potential before terminally differentiating into SGZ astrocytes. Further work is required to determine whether Gfap-positive stellate cells are indeed terminally differentiated, or whether they are a population of astroglia that, despite having different morphology to the radial cells of the young adult brain, may also be able to re-enter the cell cycle, and so represent a population of dormant progenitors. In support of this notion, stellate SGZ astrocytes also express Sox2 and are able to regulate neurogenesis within the niche (Lugert and Taylor, 2011).

The use of a range of transgenic reporter lines has enhanced the detail in which adult neurogenic niches can be analysed; however studies using different reporter lines, that ostensibly mark the same population and yet suggest contradictory results, has highlighted the huge amount of heterogeneity within populations of cells. Analysing large populations of cells to determine the fine details of fate may not be the most appropriate method. A detailed investigation to address this issue used Nestin::CreER to genetically mark progenitors, but used a significantly reduced amount of tamoxifen to induce recombination, providing the opportunity to follow the fate of individual clones (Bonaguidi *et al.*, 2011). After recombination, the majority of reporter-positive cells are radial cells: these cells can then take the choice to asymmetrically divide to give rise directly to an astrocyte, or, indirectly, through an intermediate progenitor (IP), to generate a neuron. Since IPs can go through many rounds of division, producing many cells, studies using thymidine analogues have therefore biased studies, and suggested that SGZ neurogenesis is far more frequent than gliogenesis. Studies at the clonal level reveal, in fact, that gliogenesis is substantially underestimated and occurs at a similar frequency to neurogenesis (Bonaguidi *et al.*, 2011). In addition, observing individual clones identifies the many fate decisions that radial neural stem cells can make, with examples of clones of 3 radial cells, and 2 IPs indicative of three rounds of division, while another group of clones consists of 2 radial cells, 1 neuron and 1 astrocyte, supporting three modes of division: self renewal, neurogenesis and gliogenesis (Bonaguidi *et al.*, 2011). Evidence was also found of unipotent differentiation, and differentiation over time without renewal. These data do not fit a 'disposable stem cell model'; rather, this model may characterise a subpopulation of radial progenitors with limited renewal. Regardless, it is evident from these studies that there is far more heterogeneity of, and within, progenitor populations than was previously considered (see figure 1.5) (Bonaguidi *et al.*, 2012).

The hippocampal neural stem cell niche displays many specialised features in its specific regulation and the functional outcome of new neuron production in comparison to the SVZ niche. Factors that influence neurogenesis from the SVZ have also been show to regulate neurogenesis in the SGZ, however, significant regional differences exist in the precise control of neural progenitors. It is evident that there is heterogeneity of progenitor populations as well as an incredible amount of heterogeneity within subsets of progenitors, as demonstrated by their discrepancies in potential and fate choice at each division. This is further exemplified by the specificity with which different molecular and physiological factors can regulate the two main progenitor types within the subgranular zone, radial and horizontal, highlighted below (section 1.1.4, page 37). The subgranular zone of the adult mammalian dentate gyrus provides an exceptional tool to understand the delicate control of neurogenesis in a characteristically different niche to the subventricular zone.

**Figure 1.5: Models of neural stem cell fate in the adult SGZ**



**Lugert et al. 2010**
Schematic to summarise the evidence that hes5::GFP marks quiescent (G0) radial (rNSC) and horizontal (hNSC) SGZ neural stem cells, as well activated hNSCs (G1/S). Their data support hNSC's as neurogenic, while rNSC's act as reserve NSC pool. The lineage relationship between rNSC and hNSCs is unknown (dotted line).

**Encinas et al. 2011**
Schematic to summarise the proposed 'disposable stem cell' model. Nestin::CreER marks rNSCs that are activated and have limited renewal potential, generating neurons (choice 1 and 2) before terminally differentiating into astrocytes (choice 3).

**Bonaguidi et al. 2011**
Schematic to summarise the unified model supported by clonal analysis of Nestin::CreER positive rNSCs. Quiescent rNSCs can be activated and return to quiescence. Activated rNSCs can take the choice of neurogenesis (A), self renewal (B), or gliogenesis (C).

### 1.1.4. Functional implications of adult neurogenesis

Many significant contributions have begun to elucidate the functional role of adult neurogenesis, with a particular emphasis on the dentate gyrus. However, with a more complex view of the SGZ niche now prevailing, it is crucial to understand what can influence the observed heterogeneity and the possible physiological advantages that heterogeneity may provide. One possibility may be that a mixed population of progenitors allows specific responses to specific stimuli, providing a fine-tuning of neurogenesis rather than a generic mechanism (Bonaguidi *et al.*, 2012). In this way, energy would be conserved and the neurogenic response may have a more direct impact on a specific circuit, compared with a broad neurogenic response followed by massive cell death. The specific control of progenitor subtypes is exemplified through BMPR-1A deleted mice, which lose horizontal neural stem cells through exhaustive proliferation, but retain radial neural stem cells suggesting this cell type is under compensatory regulation (Mira *et al.*, 2010). Interestingly, physical exercise can increase hippocampal expression of noggin, a BMP antagonist, thereby promoting a proliferative response, which supports physiological stimulus as a regulator of neurogenesis (Gobeske *et al.*, 2009). In accordance with this, running has been shown to selectively activate radial neural stem cells to enter the cell cycle (Lugert *et al.*, 2010), supporting the hypothesis that different physiological stimuli can induce neurogenesis from specific progenitor subtypes within the SGZ.

The ability of running to stimulate SGZ neurogenesis, has, in fact, been known for over a decade; experiments show an increase in the number of proliferating cells (thymidine analogue incorporation) and resulting neurons in runners, compared to non-runners (Van Praag *et al.*, 1999). The Morris Water Maze (MWM) is a classical approach to assess hippocampal function: measuring latency to target, and time in target quadrant, provide quantifiable measures of learning and spatial navigation, with runners performing significantly better than non-runners (Van Praag *et al.*, 1999). This poses the interesting question as to why exercise confers enhanced neurogenesis? It seems likely that exercise in the wild is coupled with encountering new environments, consisting of new habitats, food sources and threats. An enhanced spatial navigation and learning, potentially attributed to hippocampal neurogenesis, could therefore provide a survival advantage. Indeed housing rodents in environmentally-enriched (EE) conditions results in the integration of more thymidine analogue-positive neurons in the granule cell layer, as well as an increased volume of GCL and total number of granule neurons compared with standard housing (Kempermann *et al.*, 1997b). EE does not increase the number of proliferating cells, however, suggesting that its effect is not on the neural stem cell itself, but on the survival of newborn neurons. Coupling EE with running may therefore recapitulate wild conditions substantially more than standard housing. The development of appropriate test-conditions in a lab is not trivial, when one is dissecting the natural potential of a neurogenic niche.

A fascinating observation is found when comparing EE-housed mice with those that have been housed in social isolation. Social isolation results in an expansion of the neural stem cell pool, while EE appears to result in relatively higher survival of newborn neurons (Dranovsky *et al.*, 2011). Accordingly, social isolation is used to model chronic stress, in which a decrease in hippocampal neurogenesis is associated with increased anxiety and depression (Evans *et al.*, 2012). Social isolation and environment enrichment therefore bi-directionally regulate SGZ neural stem cell fate. By expanding the progenitor pool during periods of environmental deprivation, this may allow for enhanced neurogenesis when an individual encounters a social environment. This is evidence that the plasticity of the nervous system can be moderated by environmental changes, while the functional consequence of newborn neurons supports a behaviour that is appropriate to the context (summarised in table 1.3).

Senescence of an organism, the process of ageing, is coupled with observations of reduced neurogenesis in the SGZ (Kuhn *et al.*, 1996). Interestingly, voluntary exercise and EE can enhance the production of newborn neurons in ageing mice. In old mice, running is shown to increase proliferation, and consequently the proportion of new neurons, to a level comparable to young non-runners (van Praag *et al.*, 2005). The effect of environment enrichment increases the proportion of surviving newborn neurons by three-fold compared to old mice in standard housing (Kempermann *et al.*, 1998). A persisting population of neural stem cells within the SGZ therefore retains the ability to respond to the same environmental cues throughout life, albeit somewhat reduced with ageing, supporting a progressive transition to quiescence. Furthermore, EE-aged mice perform significantly better on spatial learning tasks than their standard-housed counterparts (Kempermann *et al.*, 1998), supporting a functional outcome of neurogenesis that is maintained during senescence.

**Table 1.3: Summary of the influence of environmental stimuli on adult SGZ neurogenesis**

| | Exercise / running<br>**Van Praag – 1999**<br>**Van Praag – 2005** | Environment enrichment (EE)<br>**Kempermann – 1997a**<br>**Kempermann – 1998a** | Social isolation<br>**Dranovsky – 2011**<br>**Evans - 2012** |
|---|---|---|---|
| NSC Proliferation | + | No change | + |
| Newborn neuron production | + | No change | - |
| Newborn neuron survival | + | + | - |
| Hippocampal learning task | + | + | - |

Table 1.3 summarises the influence of positive and negative environmental stimuli, including exercise, on the proliferation of neural stem cells, neurogenesis and the functional consequence. An increase or improvement is represented by '+', while a reduction is represented by '-'.

It is clear that environment and ageing significantly influences the SGZ niche to support an appropriate neurogenic response; however, a further broad factor to consider is genetic background. This is particularly important when using animal models for human neurogenesis, as the use of inbred animal lines to elucidate the response to physiological factors may not truly reflect the situation in humans. Investigations of SGZ neurogenesis in three common inbred laboratory mice strains (C57BL/6, BALB/c, 129/SVj), and a single outbred strain (CD1), reveals genetic background has a significant influence on the SGZ niche

(Kempermann *et al.*, 1997a). All strains show BrdU incorporation under standard conditions, and thus proliferation, but C57BL/6 showed significantly more incorporation than the other strains after 1day. After a four-week chase, 129/SVj mice show increased gliogenesis and half the rate of neurogenesis compared to the other strains (Kempermann *et al.*, 1997a). Genomic differences within species can, therefore, contribute to proliferation rate and cell-fate decisions (summarised in table 1.4).

While a diverse gene pool is often found in nature, the use of inbred mouse lines emphasises the different points of neurogenic regulation that exist in an outbred population. An important consideration is whether differences observed in neurogenesis reflect a difference in physiological response. While EE enhances neurogenesis in both C57BL/6 and 129/SVj lines (Kempermann *et al.*, 1997b; Kempermann *et al.*, 1998), evidence suggests the mechanism is dependent on genetic background. An increase in newborn neuron survival is observed in adult C57BL/6 mice without an observable influence on SGZ proliferation (section 1.1.3, page 38). Conversely, 129/SVj mice encounter increased proliferation in response to EE, with no effect on newborn neuron survival (Kempermann *et al.*, 1998). Thus, although the net response to stimuli is similar, the mechanism differs between inbred lines. These substantial neurogenic differences indicate that the response of the SGZ niche to physiological stimuli is dependent on genetic background (summarised in table 1.4). Such data indicate discrete ways in which outbred populations may respond to the same neurogenic stimulus. This discrepancy is a significant issue in respect to our understanding of human neurogenesis and therapeutic strategies that aim to target different features of neurogenic regulation. Here, the limitations of using inbred mice highlight the functional significance of adult SGZ neurogenesis in response to environmental enrichment, as a conserved increase in newborn neurons occurs through alternate mechanisms.

**Table 1.4: Summary of the influence of genetic background on neurogenic rate and response**

| | C57BL/6<br>**Kempermann 1997b** | 129/SVj<br>**Kempermann 1997b** | BALB/c<br>**Kempermann 1997b** | CD1<br>**Kempermann 1997b** |
|---|---|---|---|---|
| Proliferation | ++ | + | + | + |
| Neurogenesis | + | - | + | + |
| Response to EE | Increased newborn neuron survival<br>Kempermann 1997a | Increased NSC proliferation<br>Kempermann 1998b | Not investigated | Not investigated |

Table 1.4 summarises the differences in proliferation and neurogenesis in SGZ neurogenesis between mice of different genetic backgrounds. An increase in rate of proliferation or neurogenesis is represented as ‘+’, while a decrease is represented as ‘-’. The neurogenic response to environmental enrichment (EE) is also compared.

Genetic differences within species have elucidated the complex relationship between the environment and the neurogenic niche. Evolutionarily larger genomic differences that occur between species can also reveal substantial information on the function and regulation of neurogenesis. The life span of an organism is proposed to be a critical factor governing cell genesis in the SGZ. Trends observed from a variety of species studied support a decreasing neurogenic rate with increasing absolute age (Amrein *et al.*, 2011). Rodents live short, preyed-upon lives and breed relatively shortly after birth compared to longer-lived animals, such as foxes and primates. Rodents are therefore thought to exhibit more hippocampal neurogenesis, because the advantage of enhanced spatial learning could have a significant and almost immediate effect on survival, whereas the advantage of spatial learning for larger vertebrates with longer life spans is likely to be more gradual, and the lower rate reflects this. Metabolic rate is arguably the ultimate governing factor of life span, as animals with high metabolic rates live short, fast lives, while long-lived animals have a relatively low cellular metabolism. Additionally, as metabolic rate shows an age-related decline, this could explain the decrease associated with senescence in all mammals studied. Metabolic rate, and as a consequence absolute age, is therefore a strong determinant of neurogenic rate. However, these conclusions should be drawn tentatively, as they neglect many other key differences between different mammalian species.

Not only is neurogenesis governed differently across the life span of different animals, but also other distinctive temporal patterns of neurogenic regulation are observed between species. In the High Vocal Centre of songbirds, it is known that seasonal changes related to breeding heavily influence neurogenesis (section 1.1.1, page 8) (Nottebohm *et al.*, 1987). The rate of SGZ neurogenesis also correlates well with the breeding patterns of mammals. Rats and mice, which show comparatively high levels of neurogenesis compared to large mammals, are not seasonal breeders, and can breed at any time after reaching maturity. Accordingly, species with low levels of SGZ neurogenesis, such as foxes, breed only once a year (Amrein *et al.*, 2011). Breeding patterns, therefore, are supported as an influential parameter of the addition of newborn neurons in the adult brain. In support of this, prolactin, a hormone released during sexual maturation, copulation, pregnancy and parturition, has been shown to increase proliferation in the SVZ and olfactory bulb in mice (Larsen and Grattan, 2010). Furthermore, prolactin-deficient mice perform significantly more poorly in hippocampal function tests, such as the Morris Water Maze, a feature that can be rescued by prolactin infusion (Walker *et al.*, 2012). This evidence supports reproductive factors imposing regulation upon the SGZ, indeed upon all adult neurogenic niches studied to date. This is rational when we consider that the environment heavily influences adult neurogenesis, and the development of the adult niche evolved alongside natural pressures. A niche that is responsive to reproductive status could confer a survival advantage that is passed to the offspring.

Adult SGZ neurogenesis exists in an intricate relationship with physiological factors including breeding, metabolic rate, and the richness of the external environment. The number of new neurons produced in adulthood changes in response to physiological stimuli, supporting a functional consequence of their addition to neural circuitry within the dentate gyrus. While paradigms that assess spatiotemporal memory are classically used to test adult neurogenic function from the SGZ, evidence that bats exhibit excellent spatial-learning abilities in the absence of adult born granule neurons (Amrein *et al.*, 2007), allows one to conceive that the function of hippocampal neurogenesis stretches far beyond an organism's ability to navigate their surroundings. In accordance with this view, recent studies have implicated newborn neurons from the adult hippocampus in pattern separation (Sahay *et al.*, 2011a), the process by which distinct memories are formed to separate similar experiences. Conversely, pattern completion is the reconstruction of a memory from a similar experience, and is dependent on mature granule neurons (Nakashiba *et al.*, 2012). This role of adult neurogenesis in the dentate gyrus is reminiscent of the functional consequence of SVZ neurogenesis in olfactory discrimination (section 1.1.2, page 24) (Gheusi *et al.*, 2000), supporting an overarching function of both niches in differentiating experiences from one another. Enriched environment results in increased SGZ neurogenesis and pattern-separation, which facilitates discrimination between the novel environmental stimuli. Sensory deprivation results in a decrease in SGZ neurogenesis, and enhanced pattern-completion, likely an adaptive response to increase survival; previous experience of food or danger can be generalised and called upon by a similar stimulus rather than specific ones (Aimone *et al.*, 2011; Sahay *et al.*, 2011b). Intriguingly, too much pattern-separation could result in a maladaptive response of an excessive attention to detail, such as that seen in autism spectrum disorders (ASD), while excessive pattern-completion and generalisation may be associated with post-traumatic stress disorder (Sahay *et al.*, 2011b). Considering the significant role adult neurogenesis has in pattern-separation, further research will address its involvement in the aetiology of ASD. What is becoming increasingly clear, however, is the critical importance adult neurogenesis has in mapping our experiences in order to maximise survival.

An intriguing and recent study uses radioactive-carbon dating, similar to archaeological techniques, to retrospectively birth-date neurons in post-mortem human tissue (Spalding *et al.*, 2013). Spalding *et al.* utilise radioactive $^{14}$C from the nuclear bomb testing period of 1955-1963 to date mark DNA in adult born cells, and provide evidence that adult neurogenesis in the human SGZ continues into middle and old-age. Strikingly, the study suggests that while only 10% of hippocampal neurons are subject to replacement in the mouse, the majority of human dentate gyrus neurons are exchanged over time, corresponding to 700 new neurons per day throughout adult life (Spalding *et al.*, 2013; Kheirbek and Hen, 2013). This important study contrasts with previous research that suggested the rate of human adult neurogenesis is too low to be of functional significance (Kheirbek and Hen, 2013), and adds substantial evidence that adult-born hippocampal neurons contribute to behaviours, personalities and psychiatric/neurological disorders (Mu and Gage, 2011). This research supports a new frontier that considers the influence of adult neurogenesis upon neural circuitry to hold significance over our cognitive processing and to be a substantial target for psychiatric therapies.

While a gross decline in neurogenesis is associated with ageing in many mammals, it is becoming clear that this is attributed to a transition to quiescence, and this in turn can be restored by exercise and environmental enrichment. Indeed, emerging evidence suggests the extent of age-related quiescence may be species-dependent. Further, it appears that the dynamic environmental landscape that has shaped evolution has modified intra-species and inter-species hippocampal neurogenic regulation, a feature that is vital to experience-dependent learning and cognitive performance. The plastic features of newborn neurons are central to the requirement, function and success of adult neurogenesis. Addition of immature neurons to existing circuitry ensures a level of flexibility that mature neurons cannot provide alone, for example through increased synaptic strength and pruning. In agreement with this, dysfunction of neurogenesis can have profound pathological and psychological consequences. Studies have shown that despite the neurogenic niches’ particularities, there are many parallels between the SGZ and SVZ in both regulation and function. Research into both niches contributes hugely to our full understanding of adult neurogenesis, where divergences and consistencies are equally illuminating.

### 1.1.5. Remnant radial glial cells as the adult neural stem cell

The identity and characteristics of niche cells, including progenitors, neural stem cells and terminally differentiating cells are described in the sections above. The influence that general environment and other physiological conditions have upon the niche indicates neurogenesis is required in adulthood to improve the chance of survival and breeding. However, in order to absolutely understand adult neurogenic niches one must consider their origin, and question whether adult neural stem cells are a cell type determined postnatally or during development. Indeed molecular regulation of adult SVZ and SGZ niches has elucidated embryonic factors, including Shh, BMP, Fgf and Notch, as crucial to function.

During development of the cerebral cortex, the neuroepithelium consists of glial cells, termed radial glia, that have cell bodies in the ventricular zone and projections that span to the cortical ‘pial’ surface. Radial glia undergo dynamic morphological changes that are attributed to the stage of the cell cycle. Within the neuroepithelium of the ventricular zone, cells copying their DNA during S phase are found basal to the ventricle, while cells undergoing mitosis are found adjacent to the ventricle (Lemke, 2009). Tangential expansion is the process by which cells in the ventricular zone self-renew, divide symmetrically, resulting in an increase in the numbers of apically anchored progenitors, while radial expansion, asymmetric division, increases the numbers of neurons (Stahl *et al.*, 2013). Importantly, it is the radial glial cells that are the progenitors of cortical neurons that populate the cerebral cortices through neurogenesis (Malatesta, 2000), and in addition, generate basal progenitors that establish the subventricular zone (Stahl *et al.*, 2013). Radial glia can be identified by single primary cilia that contact the circulating CSF, and a radial projection to the pial surface. This projection serves as migratory scaffolding for newborn neurons to travel along in order to occupy their respective cortical layer, while progenitors also migrate along the projection to generate a new germinal layer, the subventricular zone (Kriegstein and Noctor, 2004; Noctor *et al.*, 2004; Lemke, 2009), further increasing the rate of neurogenesis (summarised in figure 1.6).

In the adult canary, in the lateral ventricle adjacent to the HVC, regions of proliferative cells within the ventricular zone are observed in clusters, termed ‘hot spots’ (Alvarez-Buylla *et al.*, 1990). Interestingly, the clusters are located in

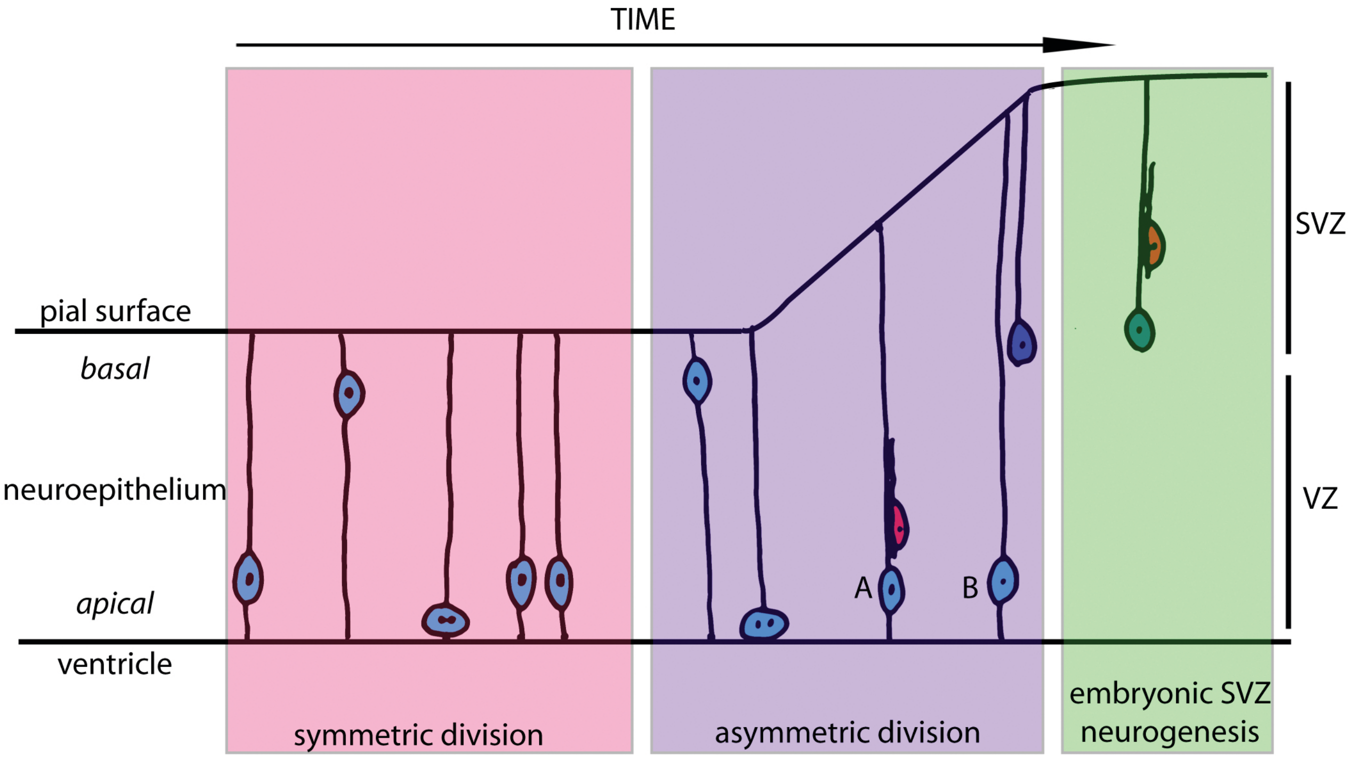


**Figure 1.6: Development of the cerebral cortex**

The schematic summarises the process of symmetric division, asymmetric division and SVZ neurogenesis from embryonic basal progenitors. Radial glial cells in the neuroepithelium undergo S phase of the cell cycle in the basal neuroepithelium, and mitose adjacent to the ventricle to generate two identicle radial glial cells. After the tangential expansion by symmetric division, radial glial cells undergo asymmetric division generating a neuroblast (A) or a basal progenitor (B). Neuroblasts from radial glial parents use the process to migrate to the appropriate cortical layer where they differentiate into neurons: the initial neurogenic phase. Basal progenitors establish a proliferative zone basal to the ventricular zone, termed the subventricular zone (SVZ). Basal progenitors also undergo neurogenesis to populate the cortices.

the same areas as dense regions of radial glia cells; whose long apical processes contact the underlying nuclei ‘en passant’. Studies addressing whether radial glia are indeed the neural progenitors in the avian brain analysed chased-tissues, after 3HT incorporation. Colabelling with the radial glia marker, vimentin, revealed 3HT-positive vimentin-positive cells, supporting radial glia as the neural progenitor (Alvarez-Buylla *et al.*, 1990; Alvarez-Buylla, 1992; Alvarez-Buylla and Kirn, 1997). These studies suggested that radial glia cells divide, that subsequent progeny lose their apical process and migrate along the process of the parent radial glia, as observed in the development of the cerebral cortices.

The conserved dynamics for cortical neurogenesis in development and adulthood in the canary suggests that radial glia present during embryogenesis remain present in discrete clusters, maintaining their neurogenic potential. This has revived the question of whether identified neural stem cells in the SVZ or SGZ of the mammalian brain similarly derive directly from an embryonic radial glial cell. Destruction of proliferative cells with Ara-C leaves two different surviving populations of niche cells, B1 cells and ependymal cells, each with cilia, a characteristic of radial glia (Doetsch *et al.*, 1999a). As outlined above, experiments have shown that B cells can incorporate thymidine analogue and give rise to other niche cells, while ependymal cells cannot (section 1.1.2, page 17). In support of the idea that B cells do derive from radial glia, B1 cells have only a single short cilia analogous to primary cilia of neuroepithelial cells during development (Mirzadeh *et al.*, 2008). Ependymal cells, conversely, have multiple long cilia supporting cilia as a distinguishing feature of niche cells (Spassky *et al.*, 2005).

Long cilia are known to have kinetic roles in the propulsion of CSF through ventricles, while primary cilia are instead involved in cell signalling propagation: the short cilium protrudes into the ventricular environment where it is able to detect changes in CSF composition. Indeed, primary cilia are crucial for canonical Shh signalling; patched, the receptor of sonic hedgehog, is present on the cilium, binding of the ligand and receptor derepresses smoothened, allowing it to translocate to the cilia where it activates Gli transcriptional activators of Shh targets (Dessaud *et al.*, 2008). Considering that intracellular transduction of Shh requires the primary cilium, and that Shh signalling is important in maintaining the adult neural stem cell pool (Han *et al.*, 2008), the presence of a single short cilium on B1 cells supports radial glial origins.

Similarly to the canary, B1 cells of the adult mammalian SVZ are observed in hotspots along the lateral ventricular wall (Mirzadeh *et al.*, 2008). B1 cells, along with two distinct ependymal cell populations, make apical contacts with the ventricular surface, with both ependymal cell populations having more than one long cilium. Interestingly, ependymal cells surround B1 cell clusters in a 'pinwheel' architecture (Mirzadeh *et al.*, 2008), a feature that appears comparable to the neural rosettes observed when neural progenitors are generated from embryonic stem cell (ESC) cultures in-vitro (Boroviak and Rashbass, 2011). Such architecture is attributed to the apical-polarity determinant, Crumbs2, in cultures, and is a required factor in the differentiation of neurons from progenitors. The comparable morphology of adult SVZ pinwheels and neural rosettes in ESC cultures is further support for adult neural stem cells as a developmentally defined cell-type whose apical signalling cascades are vital for the determination of neurons. Future studies may elucidate the role that apical-polarity proteins play in the pinwheel architecture observed in-vivo, and whether they are required for the neurogenic potential of B1 cells.

Additional evidence for the radial glial origin and identity of B1 cells in the adult SVZ has been demonstrated using transgenic rats to mark and follow radial glia (Merkle *et al.*, 2004). At postnatal day 0 to 4, the radial glial cell marker, RC2, is present in morphologically-identical radial cells in the ventricular zone of the lateral ventricles; however after day 4, RC2-positive cells appear to retract their processes and downregulate RC2, while Gfap-positive cells increase. Local injection of an adenovirus, expressing Cre-recombinase, results in reporter-positive radial glia at P0 and labelling of subsequent progeny. By postnatal day 10, GFP is found in cells within the olfactory bulb, RMS and SVZ niche, supporting P0 radial glial cell marker RC2-positive cells as multipotent stem cells (Merkle *et al.*, 2004). Concurrently, fluorescence activated cell sorting (FACS) of GFP-positive cells reveals their potential for long term passaging as neurospheres, and differentiation into neural lineages. These data support radial glial cells as the precursor of SVZ B1 cells. Importantly, recent studies have identified that B1 cells can retain a long, Gfap-positive, basal process that extends endfeet on to blood vessels and contributes to gliotubes (Mirzadeh *et al.*, 2008), down which neurons migrate, reminiscent of the radial processes that function as scaffolding in the developing cerebral cortex.

Cells with radial processes and radial glial expression profile are also present in the dentate gyrus of the hippocampus, similarly suggesting a retention of radial glial identity. Research has shown that granular cells of the dentate gyrus arise through three germinal matrices: a primary germinal matrix that consists of proliferative cells in the subventricular zone of the embryonic lateral ventricles, which migrate inward to form a postnatal secondary germinal matrix, and the developing dentate gyrus close to the pial surface (Kempermann, 2006). The radial glia cell bodies within this matrix become confined to the subgranular zone of the dentate gyrus, termed a tertiary germinal matrix, in juvenile and adult life. The sequential migration and restriction of radial glia, from embryonic stages through to adult, results in cell bodies with processes extending in different orientations.

Due to the large amount of morphological change that occurs in the developing hippocampus, a recent study has performed detailed lineage-tracing studies to determine the embryonic origin of neural stem cells in the adult SGZ. Previous research has identified crucial roles for primary cilia and sonic hedgehog (Shh) signalling during development of the hippocampus in maintaining a neurogenic niche into adulthood, exemplified by embryonic ablation of ciliary genes or the Shh receptor, smoothened, preventing postnatal SGZ neurogenesis (Han *et al.*, 2008). In addition, Shh-responsive cells have radial glial processes during development of the hippocampus (Li *et al.*, 2009). By marking Shh responsive cells and fate-mapping their progeny with Gli1::CreER$^{T2}$, Li *et al.* (2013) argue that progenitors in the embryonic ventral hippocampus relocate to the dentate gyrus, where they are the major contributors to the neural stem cell population in the SGZ niche (Li *et al.*, 2013). Taken together, these studies provide confirmation of the requirement for Shh signalling and primary cilia in generating and maintaining the SGZ niche, and additionally, build on the traditionally defined germinal matrices origin of SGZ neural stem cells. Evidence supports a radial glial origin to adult SGZ neural stem cells, and future studies may determine whether the high level of heterogeneity within the adult niche is the consequence of different origins or an inherent genetic mosaicism, as suggested by Li *et al.* (2013).

**Table 1.5: summary of evidence to support radial glial origins and characteristics of adult neural stem cells**

| | SVZ | SGZ |
|---|---|---|
| Basal projection | **+**<br>Mirzadeh 2008 | **+/-**<br>Lugert 2010 |
| Primary cilia | **+**<br>Mirzadeh 2008 | **+**<br>Han 2008 |
| Lineage-tracing | **+**<br>Merkle 2004 | **+**<br>Li 2013 |

Table 1.5 summarises the evidence of the radial glial origins and characteristics of adult neural stem cells in the SVZ and SGZ. Positive results are represented by ‘+’, while negative data is represented by ‘-’.

In conclusion, these data support populations of embryonic radial glia persisting and comprising adult neural stem cell populations (summarised in table 1.5). The presence of a neurepithelial-like primary cilium, the radial glial origin, and presence of a long basal process is highly indicative that adult SVZ B1 cells are a remnant population of embryonic radial glial cells, that maintain their potential. This is further supported by an in-vivo pinwheel architecture similar to that observed in the direct progression of ESCs to neural progenitors in culture. Furthermore, the presence of a primary cilium on embryonic hippocampal progenitors, and their temporal progression to occupy the SGZ supports primary cilia and sonic hedgehog signalling as a shared feature in the development and maintenance of both these classic niches. The traditional view that describes gross morphological changes around radial glia populations from the SVZ of the embryonic lateral ventricles to form the dentate gyrus and maintain a proliferative SGZ in the adult is supportive of a radial-glial identity of radial neural stem cells, and recent lineage-tracing data corroborate the sequential migration of progenitors to the SGZ. The origin and identity of adult neural stem cells is a fundamental branch of our understanding of neurogenesis as a whole, and is invaluable to the integration of scientific fields of developmental and clinical biology in the biomedical aspirations of novel diagnostics and therapeutics aimed at neurological disorders and brain tumours.

### 1.1.6 Neurogenesis described in the adult mammalian hypothalamus

Since the discovery of adult neurogenic niches in the SVZ and SGZ of the mammalian brain, additional regions with neurogenic potential have been identified in many other regions of the central nervous system, including the hypothalamus, amygdala, cortex, and spinal cord (Gould, 2007; Barnabe-Heider *et al.*, 2010). The hypothalamus is a major neurosecretory centre of the brain, situated in the ventral diencephalon, where it regulates developmental, physiological and psychological processes fundamental to viability and survival.

In the vertebrate, the hypothalamus surrounds the third cerebral ventricle and exploits its privileged position, adjacent to fenestrated capillaries, in order to detect and respond to changes in circulating factors, and to regulate the anterior pituitary. In this manner, hypothalamic circuitry can send and receive information through direct innervation with other brain areas, as well as directly modulate endocrine factors that have a more global influence. It is for this reason that the hypothalamus is crucially implicated in homeostatic mechanisms such as energy and fluid balance, circadian rhythms, the stress response, as well as growth and reproductive behaviours (see figure 1.7). The hypothalamus is therefore positioned at the crux of an organisms' relationship with its environment and ensures the appropriate response to a dynamic world. In accordance with this notion, conserved cell-types based on function and expression profiles are observed in annelid worms, Nereididae (Tessmar-Raible *et al.*, 2007), supporting a hypothalamic ancestry that dates back to the common evolutionary origin of vertebrates and worms.

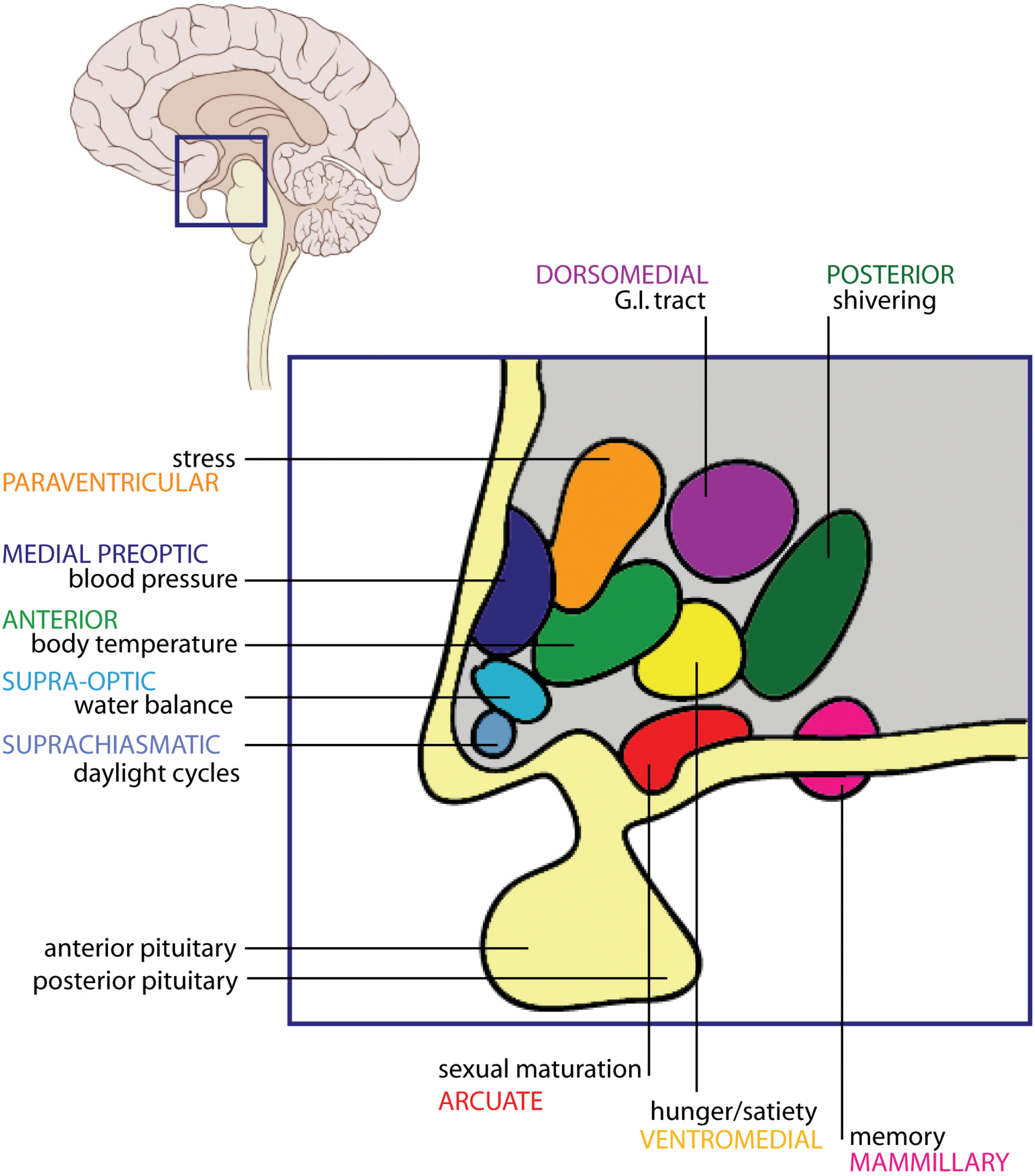


**Figure 1.7: Hypothalamic nuclei and function**
The cartoon shows the location of the hypothalamus within the human brain, and the position of nuclei within the hypothalamus. The names of the hypothalamic nuclei are colour-coded adjacent to their general function. Note: nuclei are often involved in the homeostatic regulation of more than one function.

Considering that adult neural stem cells in other brain regions are heavily influenced by environmental factors, and themselves are maintained within a rich microenvironment, the proximity of circulating CSF and blood to the hypothalamus, as well as its evolutionarily conserved relationship with the external environment, present the adult hypothalamus as a prime location for a potential neurogenic niche in mammals. Strong evidence to support this idea was first provided through studies in rats. Infusion of the mitogen, brain derived neurotrophic factor (BDNF), with thymidine analogue, BrdU, into the third (hypothalamic) ventricle of the adult rat resulted in an increased number of hypothalamic cells that incorporated BrdU during S phase, compared to infusion of saline alone (Pencea *et al.*, 2001). A significant number of BrdU-positive cells were observed to express neuronal markers and show neuronal phenotypes, suggesting that BDNF stimulated proliferation and neuronal differentiation from a potentially local, undefined niche.

Subsequently, progress was made in identifying hypothalamic neurogenesis in birds. Similar to the investigation of the relationship between canary neurogenesis and seasonal mating (section 1.1.1, page 9), studies investigated the potential role of hypothalamic neurogenesis in the courting behaviour of ringdoves (Cheng, 2013). Electrolytic lesion of the male hypothalamic ventromedial nuclei (VMN) resulted in impaired courtship behaviour, which was proceeded by proliferation (BrdU incorporation) in the ventricular zone of the third ventricle, adjacent to the lesion site (Chen *et al.*, 2006). Eight weeks post-lesion, BrdU positive neurons were observed in the VMN and males restored courtship behaviour, suggesting regenerative neurogenesis. While this is not direct evidence of a physiological role for adult hypothalamic neurogenesis in the uninjured dove, it does support a local neural progenitor population in vertebrates that can be activated to restore function.

Further mammalian studies have found that, in stark contrast to the traditionally defined neurogenic niches of the SVZ and SGZ, hypothalamic proliferation is relatively infrequent, though constitutive (Kokoeva *et al.*, 2007). This feature has concealed the identity of hypothalamic adult neural stem cells, and shrouded their existence in uncertainty. Despite this, the classic in-vitro methodology of neurosphere formation supports the presence of a neurogenic niche within the hypothalamus, and furthermore, provides an insight into the identity of the neural precursor. In a study by Xu et al. (2005), cellular dye, DiI, was injected into the fourth ventricle of adult rat brains in order to label cells lining the third ventricle. Using Fluorescence-Activated Cell Sorting (FACS) of dissociated hypothalamic tissue, it was observed that DiI-positive cells could form neurospheres, whose formation could be enhanced by the addition of basic fibroblast growth factor (bFGF). In vivo, injection of bFGF directly into the third ventricle resulted in increased BrdU incorporation in Gfap-positive cells of the ependymal layer of the third ventricle. Moreover, four weeks later, BrdU-positive neurons could be identified, providing evidence for a neurogenic cell type within the ependymal lining of the third ventricle (Xu *et al.*, 2005). Notably, infusion of insulin-like growth factor-1 (IGF-1) results in a selective incorporation of BrdU in the central region of the third ventricle ependyma, dorsoventrally (Perez-Martin *et al.*, 2010). Taken together, these data suggested the existence of a possible hypothalamic adult neural stem/progenitor cell; located in the ependymal layer of the third ventricle. However, the identity of the niche cells involved, as well as the neural stem/progenitor cells themselves, remained widely contested.

The ventricular zone of the third ventricle is unique amongst the ependyma of the central nervous system. Lining the anterior and dorsomedial third ventricle, ependymocytes exist as an epithelial-like cell type forming a barrier to the CSF, as in the SVZ. Intriguingly however, the ventromedial and posterior ventricle is comprised of an ependymal cell-type exclusive to the hypothalamus, the tanycyte (Rodriguez *et al.*, 2005). Reminiscent of embryonic radial glial cells, this cell-type is structurally defined by a cell body within the ventricular zone and an elongated basal process. Different tanycyte sub-populations are defined based on process projection: the beta-tanycytes project to the median eminence, while alpha-tanycytes extend basal projections to the hypothalamic nuclei. Historically, the different tanycyte subtypes have been defined by their dorsoventral position, and despite a morphological similarity, these unique cells show remarkable heterogeneity in function (see figure 1.8).

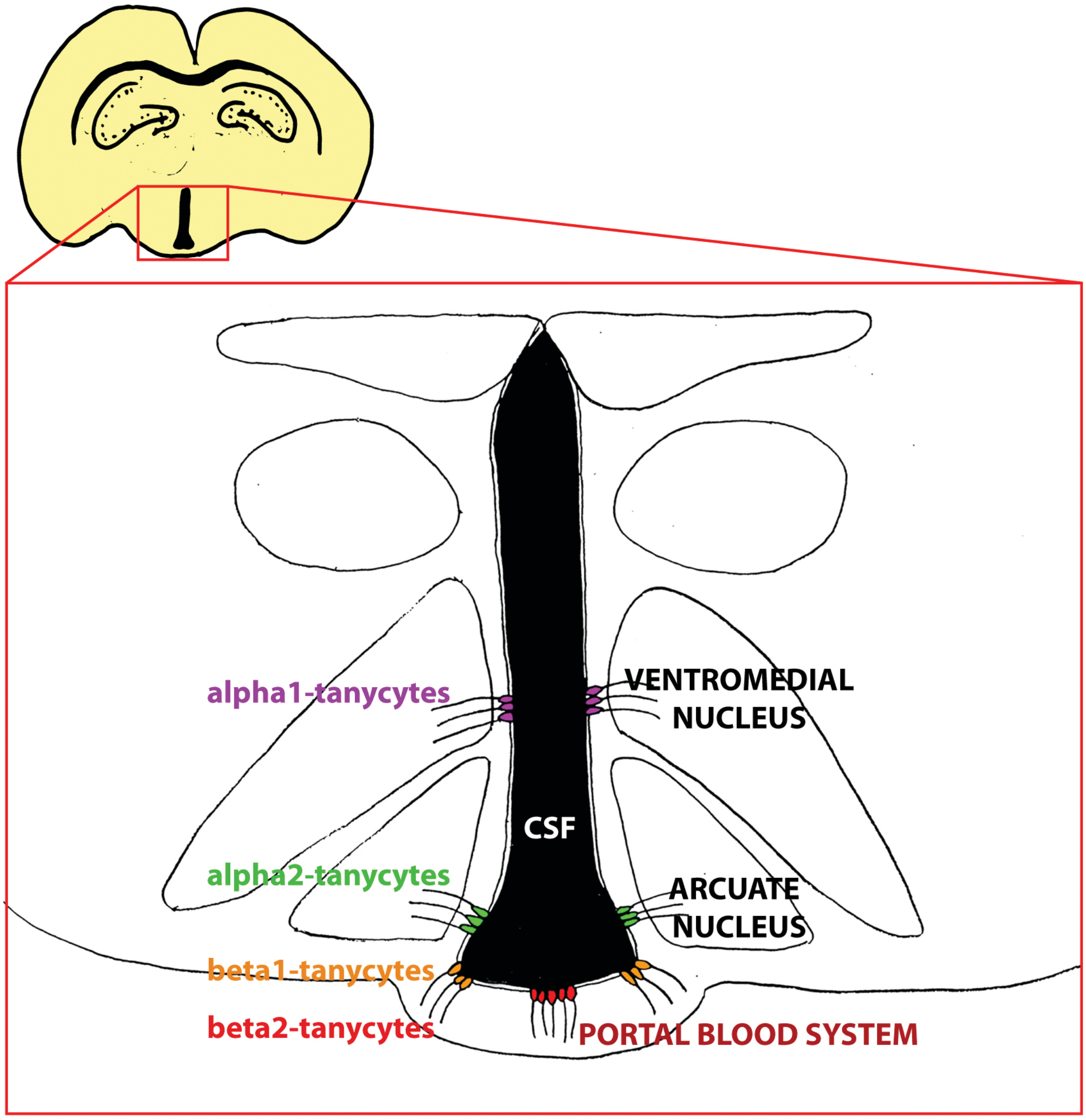


**Figure 1.8: Hypothalamic tanycyte subtypes**
A schematic of the tuberal hypothalamus is shown, enlarged from a cross section of the adult mouse brain. The cartoon indicates the relative position of tanycyte subtypes (α1, α2, β1, β2) along the dorsoventral axis of the 3rd ventricle, and their projection targets (ventromedial nucleus, arcuate nucleus and portal blood system respectively). Beta-tanycytes have barrier properties, alpha-tanycytes allow diffusion across ventricular wall. Tanycytes as bi-directional conduits aids transport of factors between CSF, hypothalamic nuclei and the portal blood system (linking the anterior pituitary).

## **Tanycyte cell function**

As a circumventricular organ, studies have succeeded in defining the barrier properties of hypothalamic tanycytes. Beta-tanycytes, at the level of the median eminence, express tight-junction proteins in an organised and regular pattern between cells (Mullier *et al.*, 2010). This feature ensures that blood-borne molecules are unable to pass directly into the CSF and brain parenchyma, and that conversely, factors in the CSF are prevented from diffusing into portal capillaries; at the same time, axon terminals and tanycyte endfeet within the median eminence are able to contact, and govern the release of neurohormones into the portal-blood system (Prevot *et al.*, 2010b). Conversely, unorganised junctions are observed between alpha-tanycytes, adjacent to the arcuate and ventromedial nuclei, enabling factors in the CSF to diffuse through the ependymal layer at these levels (Mullier *et al.*, 2010). These barrier properties of tanycyte subtypes are crucial to the success of the hypothalamus, ensuring that communication is permissible between brain and body, whilst reducing the risk of transmitting blood-borne infection to the CNS. Further, tanycytes function as transport links between hubs of information, as their processes provide bidirectional conduits for factors between the CSF, hypothalamic nuclei and the portal blood system (Rodriguez *et al.*, 2005). In this way, tanycytes can be considered to be master regulators of the brain-body communication network (see figure 1.8).

Such a ‘master regulator’ role is exemplified by the identified role of tanycytes in neuroendocrine modulation of Gonadotropin-releasing hormone (GnRH), the master regulator of sexual maturation and reproduction. Synthesised by GnRH neurons in the tuberal hypothalamus and secreted at nerve terminals in the median eminence, the neurohormone enters the portal-blood system and travels to the anterior pituitary. GnRH stimulates anterior pituitary gonadotropes to produce gonadotropins: follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which are essential endocrine regulators of development, growth and puberty. In females, surges of FSH and LH release are a prerequisite to ovulation, while a tightly controlled physiological balance of such hormones is required for the correct progression of the oestrous cycle and pregnancy (Martini and Nath, 2009). Careful studies have given evidence to the direct, morphological interaction between GnRH neurons and tanycytes, demonstrating that during diestrous, GnRH nerve terminals are embedded within tanycyte endfeet in the median eminence (Prevot *et al.*, 2010b; Prevot *et al.*, 2010a). Remarkably, the authors observe a retraction of tanycyte processes during proestrous, creating a pericapillary space that provides a route for the secreted hormone to enter the portal blood system (Baroncini *et al.*, 2007). Oestrogen released from ovarian follicles is considered to be the stimulus to drive this dynamic change in tanycyte structure that ultimately results in a proestrous surge of GnRH, and consequently, a preovulatory surge in FSH and LH, followed by ovulation (de Seranno *et al.*, 2010). The cooperative action between tanycytes, neurons and the endocrine system support a physiological process crucial to life, seemingly dependent on the morphological plasticity of a unique population of ependymal cells.

Oestrogen-induced tanycyte-remodelling is one strategy the endocrine system employs to maintain homeostatic control of reproduction. Interestingly, studies in the hypothalamus of the Djungarian hamster, a seasonal breeder, provide evidence that photoperiod can influence the characteristics of tanycyte processes (Kameda *et al.*, 2003). When hamsters are housed in constant darkness, a complete loss of processes is observed, in contrast to the high density of processes in constant light, suggesting tanycyte remodelling in response to photoperiod may be a significant mechanism in seasonal breeders (Kameda *et al.*, 2003; Bolborea and Dale, 2013). The association between season/photoperiod and adult neurogenesis has been implicated in songbirds (section 1.1.1, page 9), where the HVc architecture is remodelled accordingly (Nottebohm, 2004). As this relationship is known to exist in vertebrates, it supports a link between seasonal breeding, adult neurogenesis and tanycytes in murine models.

The tanycytes' intricate interaction with the endocrine system extends beyond reproduction to other homeostatic processes, such as those under thyroid regulation, including growth and metabolism. Type 2 iodothyronine deiodinase (D2), involved in the generation of thyroid hormone from thyroxine, is highly expressed in tanycytes, allowing these cells to produce thyroid hormone locally within the central nervous system (Lechan and Fekete, 2007). Fasted animals express increased levels of D2 in the tanycyte-rich region, while animals treated with alloxan, to kill tanycytes, show impaired feeding behaviour (Sanders *et al.,* 2004), providing support to tanycyte regulation of the homeostatic control of energy balance.

A role for hypothalamic neural stem cells in energy homeostasis is similarly supported, by the observation that inducing local proliferation with ciliary neurotrophic factor (CNTF) leads to a decrease in the body-weight of mice, which is maintained long-term (Kokoeva *et al.*, 2005). The energy balance circuit is primarily regulated by the hypothalamic arcuate nucleus, which consists of neurons that contain appetite-modulating proteins and that project to different hypothalamic nuclei. Agouti-related protein (AGRP) and neuropeptide Y (NPY) are both orexigenic and stimulate feeding, while pro-opiomelanocortin (POMC)-containing neurons act to suppress appetite and inhibit feeding. Degeneration of AGRP neurons leads to an increase in hypothalamic proliferation, and a regeneration of the lost neurons (Pierce and Xu, 2010), supporting the presence of a local neural precursor population that can be activated to replace physiologically responsive neurons.

Inhibition of proliferation is a useful tool to interpret the role and requirement of newborn cells, and has been used to interrogate the classical adult neural stem cell niches (section 1.1.2, page 17; section 1.1.3, page 28). When Ara-C is used to block proliferation in the hypothalamus of CNTF-infused mice, they do not maintain reduced body weight, and increased feeding is observed (Kokoeva *et al.*, 2005). This supports a neurogenic population whose progeny have a functional consequence upon the neural circuitry controlling food intake. Indeed, while NPY and AGRP neurons are generated at embryonic day E10.5 in mice, studies of these neurons between 4 and 12 weeks of postnatal age observe a replacement of embryonic-born neurons with those generated during juvenile life (McNay *et al.*, 2012). These data provide in-vivo support for the presence and functional relevance of a neural stem or progenitor population in adolescent mice. Interestingly, in response to a high-fat diet (HFD), known to induce apoptosis of AGRP and NPY neurons (Moraes *et al.*, 2009), the number of neurospherogenic cells increases while the rate of neurogenesis is reduced (McNay *et al.*, 2012). In contrast, Lee *et al.* (2012) observe an increase in newborn neurons in mice that are subject to the HFD regime. Furthermore, destruction of proliferative cells by irradiation reduces weight gain in HFD mice compared to those who underwent sham irradiation (Lee *et al.*, 2012a). The apparent contradiction whereby an increase in proliferation and destruction of proliferative cells can reduce weight-gain is indicative of the complex relationship between physiological stimulus and regulation of a neural stem cell niche, akin to the SVZ and SGZ.

When considering the physiological parameters that influence neurogenesis in other neural stem cell niches in the mammal, such as season and exercise, it is reasonable to consider that the hypothalamus, which controls our adaptive response to such environmental cues, is also under neurogenic governance. The widely researched example of energy homeostasis supports this through experimental data, as well as the rationale that through childhood, puberty, adulthood and old age, an organism will experience changing requirements, abundance and expenditure of energy. While synaptic plasticity of the neural circuits involved will compensate for the daily stressors, the external environment can be too extreme to rely upon a relatively fixed network for survival. Adult neurogenesis would provide a plasticity of the networks, allowing long-term adaptation of an organism in parallel with the changing metabolic rate and demands of the environment.

### Tanycytes as hypothalamic neural stem cells

In an attempt to identify a hypothalamic neural stem cell niche, researchers have utilised transgenic mice that express a reporter specifically in Nestin-positive cells and any progeny, previously used to elucidate neural stem cell populations in the SVZ and SGZ. Initial observations with a Nestin::GFP transgenic line demonstrate that tanycytes express the reporter, and neurospheres generated also express the reporter, suggesting that tanycytes may be a significant component of the hypothalamic niche (Bennett *et al.*, 2009). Similarly, by inducing recombination of Nestin::CreER mice postnatally, and analysing the lineage of tanycytes after one month, Lee *et al.* (2012)

observe reporter-positive neurons in the median eminence, arguing that beta-tanycytes are a neurogenic population in adolescent mice (Lee *et al.*, 2012a). However, while the presence of reporter-positive neurons in the median eminence may suggest they originate from the local beta-tanycytes, Nestin is a marker of all subpopulations, obscuring definitive evidence of the identity of the neurogenic cell.

While adult neurogenesis has been identified in the hypothalamus, as yet the identity of the adult neural stem cell population has remained elusive. Much evidence of neurogenesis is derived from the juvenile hypothalamus, which may be a remnant of developmental or postnatal neurogenesis, rather than true adult neurogenesis. For example, Lee *et al.* (2013) induce recombination of Nestin::CreER$^{T2}$-positive cells at postnatal day 4 and much of their analysis is performed in adolescent mice (Lee *et al.*, 2012a). Furthermore, research that has identified adult neurogenesis also observes newborn cells at a distance from the third ventricle (Kokoeva *et al.*, 2007), which could be considered too far for migration and may suggest the presence of a parenchymal progenitor. In the absence of a characterised hypothalamic neural stem cell niche, studying the contribution and function of adult neurogenesis to the homeostatic regulation of physiological processes has led to inconsistencies.

Previous studies have demonstrated that cells within the ependyma of the third ventricle can generate neurospheres in culture, a classic methodology for assessing neural stem cell potential. The hypothalamic ependyma contains specialised cell types, termed tanycytes, which show a significant degree of heterogeneity. The different tanycytes subpopulations, alpha-1, -2 and beta-1, -2, have specific barrier properties and extend projections to discrete regions. While various lines of evidence suggest a neural stem cell identity of beta-tanycytes, others direct attention to their dorsal counterparts, alpha-tanycytes. Although the stem-like characteristics between these subsets remains contested, it is increasingly clear that tanycytes of the hypothalamic third ventricle retain neurogenic properties. Moreover, studies addressing the functional implications of adult neurogenesis have identified regulatory roles in energy homeostasis, providing tantalising evidence for cellular homeostasis as central to the hypothalamic role of maintaining neural and endocrine balance in response to other physiological stressors.

A major obstacle in addressing the neural stem/progenitor properties of the hypothalamic ependyma is the lack of tanycyte subtype specific markers, despite their known heterogeneity in function. As in the SVZ and the SGZ, efforts were made to characterise the different cell types based on the expression of a number of neural stem cell markers including Nestin, Sox2 and BLBP (Xu *et al.*, 2005; Bennett *et al.*, 2009). Both Nestin and Sox2 are expressed in all ependymal cells, while BLBP is absent from tanycyte subtypes, thus not providing any further insight into the heterogeneity of progenitor characteristics. Previous studies in Placzek lab, however, have succeeded in identifying tanycytic markers that show restricted expression patterns in the different tanycyte subtypes. Careful analysis of Gfap expression revealed it is virtually exclusive to the dorsal most tanycytes of the alpha-2 subtype, with some expression observed in alpha-1 tanycytes but lacking elsewhere in the ventral ependyma (Robins *et al.*, 2013a). As previous studies in rats had provided evidence that bFGF could stimulate Gfap-positive hypothalamic ependymal cells to incorporate BrdU (Xu *et al.*, 2005), further progress was made in characterising expression of Fgfs local to the hypothalamus.

Extensive studies in Placzek lab identified two endogenous Fgfs with restricted expression patterns within the mouse hypothalamic ependyma. Specifically, beta-tanycytes and the ventral population of alpha-2 tanycytes express *Fgf10,* while *Fgf18* is detected in ventral alpha-2 tanycytes alone (see figure 1.9) (Robins *et al.*, 2013a). In both mouse and human, the Fgf family comprises 23 members that bind to one or more of the four Fgf receptors to activate downstream signalling factors. The large number of ligands compared to the relatively small number of different receptors results in a significant degree of compensation between ligands, with many ligands sharing the same receptor (Zhang *et al.*, 2006). Indeed both Fgf10 and Fgf18 share receptors with bFgf (Fgf2), although not with each other (Mason, 2007). Furthermore, Fgf10 and Fgf18 have essential roles during development, mutant mice generated to lack either of these Fgfs results in craniofacial defects (Wan *et al.*, 2009). Mice

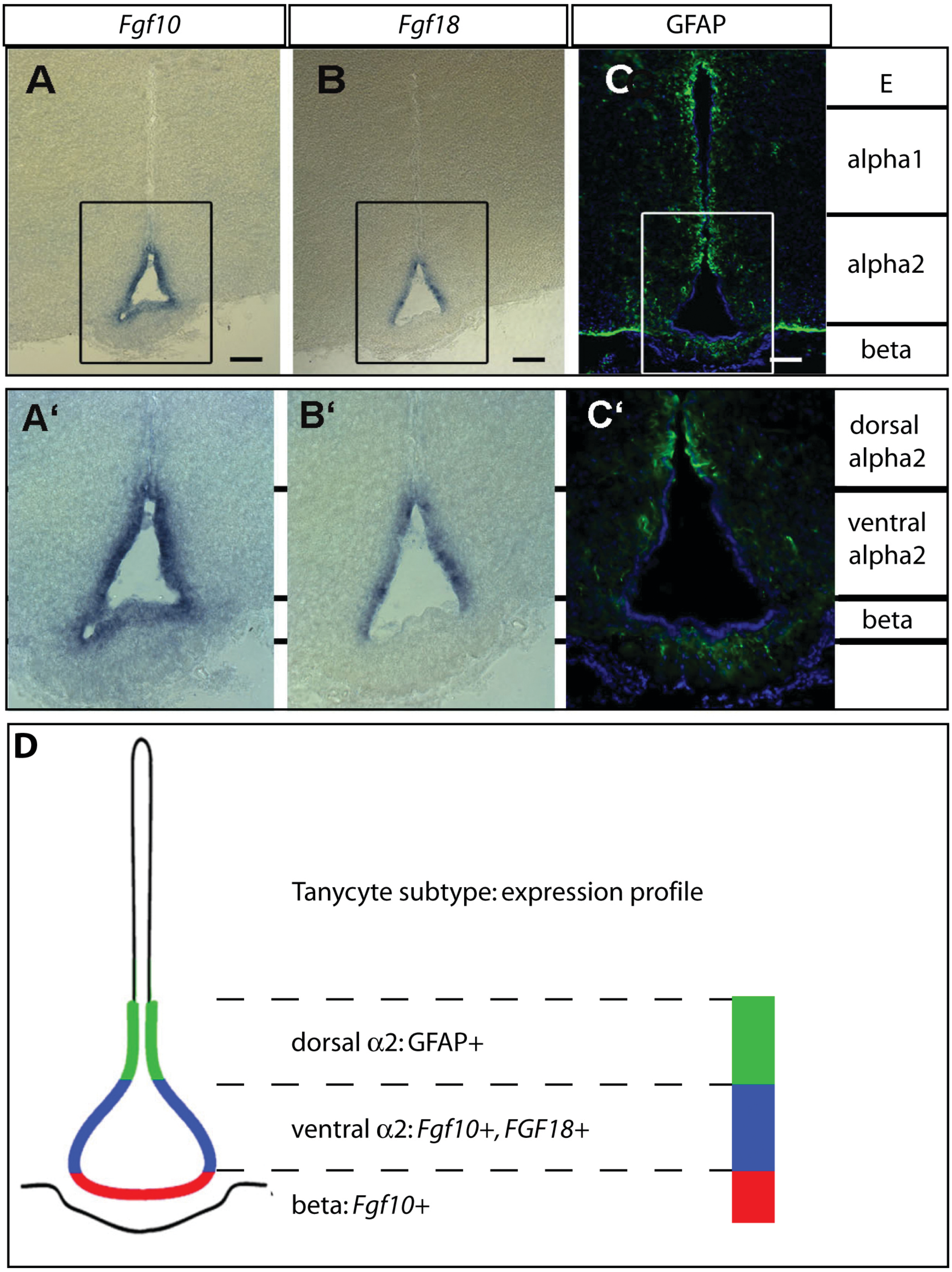


**Figure 1.9: Expression profile of tanycyte subtypes**
The figure shows the expression profile of glial fabrillary acidic protein (GFAP) restricted to dorsal alpha2-tanycytes (C'), with varying levels in alpha1-tanycytes and ependymocytes/E (C). mRNA of endogenous FGF10 is detected in ventral alpha2-tanycytes and beta tanycyte subtypes (A, A'), while mRNA of endogenous FGF18 is detected in the ventral alpha2-tanycyte subtype alone (B, B'). A schematic summarises the expression profile data (D). This figure is reproduced from Robins *et al.* (2013) with permission from authors.

deficient in Fgf18 have widespread defects in cartilage and bone formation due to dysregulation of proliferation, and the ligand has also been connected to the control of pituitary progenitor cells during embryogenesis (Haque *et al.*, 2007). Similarly, Fgf10 is involved in the vascularisation of the posterior pituitary (Liu *et al.*, 2013) and controls the transition of neuroepithelial cells to radial glia before the onset of embryonic neurogenesis (Sahara and O'Leary, 2009). Together, these data implicate Fgf10 and Fgf18 as regulators of hypothalamic progenitor cells and provide initial evidence of tanycyte subtype heterogeneity at the molecular level, with respect to progenitor characteristics.

While recent efforts have made significant progress in identifying hypothalamic neurogenesis and recognising the neurogenic potential of the hypothalamic ependyma, a considerable number of questions remain. The potential of the different tanycyte subtypes has not yet been addressed, nor the relationship between niche cells. The restricted expression of *Fgfs* to tanycyte subtypes suggests local function, however it is not yet known whether they have a role in the adult hypothalamic niche. In addition, while many studies have indicated a role for adult hypothalamic neurogenesis in energy homeostasis, the potential of the niche under standard housing conditions has not been thoroughly explored, nor its response to other physiological stimuli of hypothalamic-regulated processes. Furthermore, unlike in the SVZ and SGZ, the developmental origin of an adult hypothalamic neural stem cell has not been investigated.

## 1.2 Thesis aims and hypothesis

I hypothesise that alpha tanycytes comprise a population of self-renewing, multipotent stem cells in the adult hypothalamus, which undergo Fgf-dependent neurogenesis in response to appropriate physiological stimuli.

In order to address the hypothesis, I will first analyse the protein expression profile of the embryonic hypothalamus, through to the adult, to investigate the origin of alpha-tanycytes and determine whether embryonic radial glial cells are represented here, as in the SVZ and SGZ. I will then perform lineage-tracing analysis on Glast::CreER$^{T2}$ mice (Mori *et al.*, 2006), which expresses the reporter protein in alpha-tanycytes after recombination. This will ensure a level of specificity that has previously not been attained in the hypothalamus. By performing careful co-localisation studies of the reporter with neuronal and astrocytic markers, I will be able to characterise the potential of alpha-tanycytes in un-stimulated mice.

The role of Fgfs on the niche, known to stimulate proliferation in-vivo, will be thoroughly assessed by the in-vitro neurosphere assay, and in-vivo infusion, with specific focus on the differences between tanycyte subtypes. Furthermore, I will develop an organotypic slice-culture protocol for the hypothalamus in order to generate a robust ex-vivo model that is amenable to manipulation, efficient, inexpensive and less invasive than in-vivo techniques. By utilising the organotypic slice-culture assay, I will assess the response of the alpha-tanycyte population to physiologically significant stimuli. Such progress in the adult neurogenesis field will characterise the potential of alpha-tanycytes in the long-term adaptation to physiological stressors, as well as elucidate the associated factors.

# Chapter 2

## Materials and Methods

## 2.1 Mice

All mice used in these studies are housed under standard conditions: mice are maintained in a 12-hour light/dark cycle at 350-400 lux. Temperatures range from 19-23°C, humidity is maintained at 55% (±10%) and there are 15-20 air changes per hour to maintain ventilation. Mice are given unrestricted access to fresh water and 18% protein rodent diet (Teklad; Harlan).

### 2.1.1 Embryonic mice

Embryonic mice are C57Bl/6, except where stated, and sacrificed at E15, E16, E17 and E18 by immersion in ice cold L-15 and decapitation of the head, according to schedule 1 practice.

### 2.1.2 Adult mice

All adult mice used in this study are female C57Bl/6, except where stated. 6-12 week old mice are used for adult tissue. For fresh tissue, animals are sacrificed using a lethal overdose of inhaled isoflurane anaesthetic (B506; Abbott) followed by cervical dislocation, according to schedule 1 practice. For fixed tissue, animals are given isoflurane anasethetic and perfused using 4% (w/v) paraformaldehyde (PFA, P6148; Sigma) in 0.1M phosphate buffer, kindly performed by Dr. Andrew Furley.

### 2.1.3 BrdU injections and infusions

For the BrdU analysis in this study, pregnant C57Bl/6 dams were injected with 50mg/kg of BrdU (B5002; Sigma) at 10mg/ml in 0.1M Tris-HCl pH 7.4. Pregnant mice were injected intraperitoneally 4 times over a 12-hour period when embryos were at E14, E15, E16 or E17. Embryos were sacrificed after 24 hours. In addition, pregnant mice were injected using the same regime when embryos were E15 and E16, offspring were then sacrificed at P0, P14, P35 or P49. BrdU treated mice and pups were regularly checked for problems and developmental abnormalities, none were observed.

For the BrdU infusions studies performed by David McNay, adult mice were stereotactically implanted with a steel cannula into the right lateral cerebroventricle, connected to an osmotic minipump and infused for 7 days. The infused solution was artificial cerebrospinal fluid containing 1μg/μl mouse serum albumin (Sigma) and 1μg/μl of BrdU (Sigma). Where appropriate, FGF2 (R&D Systems) was added to the infusion solution at a concentration of 64.8ng/μl. Infused mice were harvested acutely or after a 6-week chase.

### 2.1.4 Transgenic mice and conditional recombination

Hes5::GFP mice are a kind gift from Professor Verdon Taylor and were constructed using the method outlined in Basak and Taylor (2007). Briefly the *hes5* gene was cloned into pBS-KS cloning vector to generate pBS-Hes5. Enhanced green fluorescent protein (eGFP) was inserted into the construct to generate pBS-Hes5-GFP. This construct was subsequently injected into fertilized oocytes, transgenic founder mice were confirmed using PCR and were crossed to the C57Bl/6 background.

Glast::CreER$^{T2}$ mice are a kind gift from Professor Verdon Taylor and Professor Magdalena Gotz, and were constructed using the method outlined in Mori *et al.* (2006). Briefly, the CreER$^{T2}$-poly-A cassette was inserted into exon 2 of Glast genomic DNA. The construct was injected into a 129SVj-derived embryonic stem (ES) cell line, recombined ES cells were injected into blastocysts of the C57Bl/6 background. Glast::CreER$^{T2}$ mice were then crossed to Rosa26R (*lacZ*) or z/EG (GFP) maintained on a C57Bl/6 background. Conditional recombination was induced by intraperitoneal injection of 20mg/ml Tamoxifen (Sigma) in corn oil (Sigma). Mice were injected for 10 consecutive days at 2mgs per day, and sacrificed after 5 days, 10 days, 6 weeks or 9 months.

## 2.2 Histological techniques

The histological staining techniques described here are used and adapted for embryonic tissue, adult tissue, tissue slices and neurospheres.

### 2.2.1 Fixation of tissue

All tissues are fixed using ice cold 4% (w/v) paraformaldehyde in 0.1M phosphate buffer, except where Xgal staining was to be performed (see section 2.2.5). Adult tissues are perfused first and then the dissected brain was fixed for 24 hours at 4°C. Embryonic tissues are dissected and fixed for 24 hours at 4°C. Tissue slices are fixed for 2 hours at 4°C. Undifferentiated neurospheres are fixed for 60 minutes at 4°C, and differentiated neurospheres are fixed for 20 minutes at 4°C.

### 2.2.2 Cryosectioning of tissue

Adult and embryonic brains are washed with 1X phosphate buffered saline (PBS) (P4417; Sigma) followed by a 24-hour incubation with 30% (w/v) sucrose (S0389; Sigma) in 0.2M phosphate buffer at 4°C for cryoprotection. Tissues are then orientated for sectioning in OCT (361603E; VWR International), frozen on dry ice and stored at -80°C in Parafilm. Brains are sectioned using a cryostat (OTF5000; Bright) at a thickness of 20μm for embryonic tissue and 30μm for adult tissue. Embryonic tissue is collected directly on to superfrost slides (J1800AMNZ; Thermo Scientific) and air dried for 3 hours before immersion in PBS for immunohistochemistry, or frozen for in situ hybridization. Adult tissue is collected into PBS as floating sections for subsequent immunohistochemistry.

### 2.2.3 Immunohistochemistry

For immunohistochemistry on embryonic sections and neurospheres, a standard protocol was followed. Sections are pre-incubated in a blocking solution containing 1% (v/v) heat-inactivated goat serum (HINGS) (16210-072; GIBCO) and 0.1% (v/v) Triton (T8787; Sigma) in PBS for 1 hour at room temperature. Antibody solutions are prepared in blocking solution, and sections are incubated for 24 hours at 4°C in primary antibody solution. The primary antibody solution is removed with 3 sequential washes in PBS and incubated in secondary antibody solution for 1 hour at room temperature, protected from light. Secondary antibody solution is removed with 3 sequential washes in PBS and slides are mounted with Vectashield mounting medium containing 4',6-

diamidino-2-phenylindole (DAPI) (H-1200; Vector Laboratories) and glass coverslips (MIC3228; SLS). To ensure antibody labelling did not result in non-specific fluorescence, secondary antibodies are incubated in the absence of primary antibodies, no non-specific labeling was detected.

For immunohistochemistry on adult floating sections and adult slices, the above protocol is adapted to improve staining. Blocking solution comprised 2% HINGS and 0.5% Triton in PBS. For adult slices, washing steps are extended to 30 minutes each and all incubation steps are extended to 24 hours at 4°C. Fine paintbrushes (Series 111; Windsor & Newton) are used for delicate handling of floating sections, and antibody staining is performed in 4-well plates (10404532; Thermo Scientific).

For antibody detection of GFP, heat-inactivated donkey serum (017-000-121; Jackson Immunoresearch) replaced HINGS at the same percentage. After incubation in primary antibody solution, sections are incubated in an anti-sheep biotin, followed by DTAF-streptavidin to amplify GFP signal. For antibody detection of BrdU, sections are treated with a 2M HCl antigen retrieval step at 37°C for 40 minutes, followed by sodium citrate buffer washes.

**Table 2.1: Primary antibodies used in this study**

| 1° Antibody | Species | Dilution | Source *reference |
|---|---|---|---|
| Arl13b | Mouse IgG | 1:1000 | NeuroMab *a |
| B-Gal | Rabbit polyclonal | 1:200 | Chemicon *b |
| BrdU | Rat monoclonal | 1:250 | AbCam *c |
| B-Tubulin | Mouse IgG | 1:2000 | Sigma *b |
| Dcx | Rabbit polyclonal | 1:500 | AbCam *d |
| Gfap | Rabbit polyclonal | 1:500 | AbCam *e |
| GFP | Sheep polyclonal | 1:500 | AbD Serotec *f |
| Ghrh | Rabbit polyclonal | 1:600 | Chemicon *g |
| GR (M-20) | Rabbit polyclonal | 1:100 | SantaCruz *h |
| Nestin | Mouse IgG | 1:200 | AbCam *i |
| NeuN | Mouse IgG | 1:1000 | Chemicon *b |

| | | | |
|---|---|---|---|
| Ng2 | Mouse IgG | 1:200 | AbCam [*j] |
| pH3 | Rabbit polyclonal | 1:1000 | Millipore [*k] |
| pMAPK (ERK1/2) | Rabbit polyclonal | 1:200 | Cell Signalling [*l] |
| RC2 | Mouse IgM | 1:5 | DSHB [*m] |
| RIP | Mouse IgG | 1:10 | DSHB [*n] |
| Six3 | Rabbit polyclonal | 1:1000 | Eurogentech [*o] |
| Sox2 | Rabbit polyclonal | 1:1000 | Chemicon [*p] |
| Sox3 | Rabbit polyclonal | 1:500 | Gift [*q] |
| Tuj1 | Mouse IgG | 1:1000 | Covance [*r] |
| Vimentin | Mouse IgG | 1:100 | Millipore [*s] |

NB. The specificity of the primary antibodies, above, has not been tested in Placzek lab by deleterious studies, however most antibodies are highly cited.

**Table 2.2: Secondary conjugates used in this study**

| 2° Conjugate | Dilution | Source |
|---|---|---|
| Alexa-488 | 1:400 | Molecular Probes |
| Alexa-594 | 1:400 | Molecular Probes |
| Cy3 | 1:400 | Jackson Immunoresearch |
| Cy5 | 1:400 | Jackson Immunoresearch |
| DTAF-streptavidin | 1:500 | Jackson Immunoresearch |
| $\alpha$-sheep biotin | 1:500 | Jackson Immunoresearch |

### 2.2.4 X-Gal staining

X-gal staining is performed on neurospheres as wholemount and 20$\mu$m sections. Neurospheres are fixed for 60 minutes in lacZ fix including 1% (v/v) Formaldehyde and 0.2% (v/v) Glutaraldehyde in PBS and then washed in lacZ wash bufer 3 times for 30 minutes. Reporter expression is developed by incubation in lacZ staining buffer containing 1mg/ml of X-gal (B4252; Sigma) in the dark. After development of signal, neurospheres are washed in PBS and post-fixed in 4% PFA.

### 2.2.5 TUNEL assay

TUNEL staining was performed on wholemount sections to detect apoptotic cells. A TUNEL Apoptosis Detection Kit is used (17-141; Millipore) but the protocol is adapted for wholemount tissue according to Smith and Cartwright (1997). To improve permeability of tissue, slices are dehydrated through successive Ethanol washes and then rehydrated. Slices are incubated in proteinase K solution for 30 minutes at 37°C to enhance exposure of the DNA to TdT (terminal deoxynucleotidyl transferase) enzyme, followed by PBS washes. Slices are then washed in equilibration buffer for 5 minutes at room temperature and then placed into TdT enzyme solution for 2 hours at 37°C to label 3’-OH ends of cleaved DNA with biotinylated deoxyuridine triphosphate (biotin-dUTP). Tissue is placed into termination buffer for 40 minutes at 37°C followed by extensive PBS washes. Slices are then placed into blocking buffer for 2 hours followed by incubation in blocking buffer containing avidin-FITC for 24 hours at 4°C in the dark. Slices are washed with PBS and mounted for fluorescent microscopy. Positive controls are generated using 1$\mu$g/ml DNase for 60 minutes at 37°C to cleave DNA for TdT labelling. Negative controls do not contain TdT enzyme.

### 2.2.6 Image acquisition and analyses

Fluorescent images are captured using an AxioImager.Z1 with Apotome (Zeiss). Axiovision 4.6/4.8 software is used to view captured fluorescent images. Light microscope images of neurospheres or tissue are captured using a dissecting microscope with Q-Capture Pro 7.0 software (Q-Imaging). Images are then imported into ImageJ or Photoshop (CS3; Adobe) where exposure, light balance and contrast are slightly adjusted to generate comparable, high-quality images. Where quantifications are performed, exposures are kept consistent. The Photoshop counting tool is used to quantify positive cells where appropriate and Prism 5/6 software is used to perform statistical analyses, including t-tests and ANOVA.

## 2.3 Western blot technique

The western blot protocol was a kind gift from Robert Piggott and is used to detect changes in protein expression levels between tissue samples.

Tissues are centrifuged at 3000RPM for 5 minutes and RIPA lysisbuffer was added for a final concentration of 10% (w/v) tissue in buffer. RIPA lysis buffer comprises 50mM Tris pH8.0, 150mM NaCl, 0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate, 1% (v/v) NP-40 and protease inhibitor cocktail (11836153001; Roche Diagnostics). Tissue is homogenised in RIPA lysis buffer using a tissue homogeniser and plastic pestle. The homogenised solution is centrifuged at 13000RPM for 20 minutes and the supernatant is transferred to a fresh eppendorf. Equal volume of Laemmli buffer is added and the solution is vortexed before boiling at 95°C for 5 minutes. Laemmli buffer contains 100mM Tris pH6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 2% (v/v) 2-mercaptoethanol.

Samples are loaded into a SDS-PAGE mini-gel consisting of 10% resolving gel and 5% stacking gel, casted in the Bio-Rad casting system. 1x SDS-running buffer is added to the tank (mini-PROTEAN Cell Tetra System; Bio-Rad) and the gel is run at 100V through the stacking gel followed by 150V through the resolving gel. The gels are transferred to polyvinylidine fluoride (PVDF) membrane (IPVH10100; Merck Millipore) and electro-blotted using Mini Trans-Blot Cell (Bio-Rad) at 100V in Towbin transfer buffer; 25mM Tris, 192mM glycine, 20% (v/v) methanol. The membrane is transferred to Tris-buffered Saline Tween (TBST) after electroblotting for washing, followed by 1 hour in blocking buffer at room temperature: 5% (w/v) dried milk powder in TBST. The membrane is incubated for 24 hours at 4°C with blocking buffer containing primary antibody, followed by TBST washes and an hour in blocking buffer containing secondary antibody. The membrane is washed in TBST and Enhanced ChemiLuminescence (ECL) solution is added to react with the secondary antibody conjugated with peroxidase, emitting light detected by the ChemiDoc (Bio-Rad) imaging system. ECL solution comprises 100mM Tris pH8.5, 1.25mM luminol, 0.2mM p-Coumaric acid, 0.02% (v/v) $H_2O_2$.

## 2.4 Neurosphere culture

The neurosphere protocol used for hypothalamic tissue is adapted from Giachino *et al.* (2009), with alterations to enzymatic dissociation in order to maintain consistency with previous hypothalamic neurosphere protocols.

### 2.4.1 Dissection of hypothalamic tissue

The brain is dissected from the cranium into ice-cold Leibovitz's 15 (L-15) media (11415; GIBCO). The hypothalamus is dissected and placed into trypsin solution for 15 minutes at 37°C for enzymatic dissociation of cells. The enzymatic reaction is stopped by addition of cold trypsin inhibitor solution containing soybean (T6522; Sigma) and 50μg/ml DNase (D4527; Sigma). The solution is centrifuged at 1000RPM for 5 minutes, and the supernatant is replaced by dissociation solution containing 50μg/ml DNase and bovine serum albumin (A8412; Sigma). A 1000μl filter tip is used to mechanically dissociate the cells, followed by a repeat centrifugation step. Neurosphere media (see section 2.3.2) was added to dissociated cells, and the solution was then added to T-25 flasks (690-175; Greiner Bio-One) for bulk culture of primary neurospheres at 37°C and 5% $CO_2$ for 7 days in an incubator (MCO-18AICUV; SANYO).

For fine dissection of specific tanycyte subregions based on antigen expression and location, the hypothalamus was sliced using a vibratome (see section 2.2.4) and further fine subdissections were performed using tungsten needles. The tissue was dissociated as above and then seeded at a clonal density of 10cells/μl in ultra-low attachment plates (3473; Corning) for quantification of neurospheres after culture period.

### 2.4.2 Media and culture regime

Neurosphere media comprises DMEM/F-12 (21331; GIBCO) supplemented with penicillin/streptomycin (15140122: GIBCO), L-Glutamine (25030024; GIBCO), B27 (17504-044; Life Technologies), 5μg/ml heparin (H3149; Sigma) and customised N2, which contains IGF instead of insulin. N2 comprises DMEM/F-12 supplemented with penicllin/streptomycin, 2μM progesterone (P7556; Sigma), 3μM selenite (S5261; Sigma), 10mM putrescine (P5780; Sigma), 1mM transferrin (T0665; Sigma) and 5μg/ml IGF-1 (I8779; Sigma). Growth factors were added for a working concentration of 20ng/ml, and include EGF (PHG0311; Life Technologies), bFGF (13256029; Life Technologies), FGF10 (PHG0204; Life Technologies), FGF18 (PHG0234; Life Technologies). Where media is supplemented with N-methyl-D-aspartate (NMDA) (M3262; Sigma) final concentrations of 10μM and 100μM are used. Neurospheres were fed after every 2 nights in culture and culture length ranges from 6 days for clonal density cultures to 10 days for clonal level cultures. Culture experiments performed by Sarah Robins were 7-10 days in length and fed once. Primary bulk cultures are not fed.

### 2.4.3 Neurosphere passaging

Neurospheres are passaged by collection of all spheres and centrifugation at 1000RPM for 5 minutes. The supernatant is replaced with TrypLE solution (12604; GIBCO) and incubated at 37°C for 10 minutes followed by the addition of equal volume of cold trypsin inhibitor solution and 50μg/ml DNase. The solution is centrifuged as before and the supernatant is replaced by dissociation solution for mechanical dissociation with a 1000μl filter tip. After a further centrifugation, neurosphere media is added and a sample of the dissociated solution is pipetted into a haemocytometer. The number of cells is quantified in 0.1mm$^3$ to provide a concentration of cells. Cells are then plated at clonal density for the subsequent culture period.

### 2.4.4 Generation of clonal neurospheres

Neurospheres that had reached the fourth passage are dissociated to single cells using the method outlined in 2.3.3. A cell sorter is used to perform single cell deposition of neurosphere cells into each well of 24 x 96-well plates (3474; Corning), providing 6 repeats of 4 conditions. The neurosphere media is not modified for clonal neurospheres. Cells are cultured at 37°C, 5% $CO_2$ for 10 days, and are fed after every 2 nights in culture.

### 2.4.5 Neurosphere quantification

An inverted Leica microscope and a hand tally counter are used to quantify the number of neurospheres within each well at 4X magnification. A graticule within the microscope eyepiece is used to measure neurosphere size, or alternatively, dividing the calculated total number of cells by the total quantified number of neurospheres provides a measure of size.

### 2.4.6 Differentiation of neurospheres

Neurosphere differentiation protocol was performed to determine the multipotency of in-vitro derived hypothalamic neural stem/progenitor cells. 8-well chamber slides (177445K; SLS) are coated with 150μg/ml poly-D-lysine (P6407; Sigma) followed by 100μg/ml fibronectin (F4759; Sigma) to provide a substrate for differentiation. Neurosphere medium with 10ng/ml bFGF and without EGF is equilibrated on the slide and a single neurosphere is plated into each chamber. Differentiating neurospheres are incubated for 7 days at 37°C, 5% $CO_2$. After the culture period, differentiated neurospheres are processed for immunohistochemistry as in 2.2.

## 2.5 Slice culture

The slice culture method, for the culture of tissue on a membrane at the interface of air and media, is adapted from Stoppini *et al.* (1991). Where collagen is used, the method is adapted from Placzek and Dale (1999).

### 2.5.1 Obtaining hypothalamic slices

8-12 week old brains are dissected into ice-cold L-15 and the pia mater is gently removed from the ventral surface of the hypothalamus with #5 forceps (11254-20; Fine Scientific Tools). A scalpel (3001; Swann-Morton Ltd.) is used to dissect the hypothalamus to form a cube of tissue, which is then appropriately orientated in 3% (w/v) low-gelling point agarose (A9045; Sigma) in HBSS (H9269; Sigma) at 37°C for vibratome slicing. When the tissue is orientated correctly within a mould, placing on ice sets the agarose. The solid agarose is removed from the mould and super glue is used to attach it to a chuck, which is inserted into the stage of the vibratome (1500; Vibratome). The stage and agarose are submerged in ice-cold PBS and ice packs are placed either side to maintain the low temperature. A blade (double edge razor blade; Tesco/ASDA) is used to obtain 200μm thick tuberal hypothalamic slices anterior-posteriorly at a speed of 6 and amplitude of 7. A fine paintbrush is used to collect the slices into ice-cold L-15.

To culture the hypothalamic slices using the interface method, the slice is placed directly onto the membrane of a culture insert (PICM03050; Millipore). Any residual L-15 is removed with a pipette and the culture insert is placed into 1100μl of equilibrated slice culture media. The culture insert allows media to diffuse over tissue without submersion.

To culture the hypothalamic slices using the collagen method, collagen beds are prepared in advance. Collagen is obtained from rat-tails using the Placzek lab protocol. Collagen beds are prepared at low pH to prevent the solution from setting. 1 part 10X DMEM is added to 9 parts collagen, and 1% (v/v) 0.8M sodium bicarbonate is added to adjust the pH and set the solution moments before pipetting. 20μl of collagen solution is pipetted into each well of a 4-well plate (10404532; Thermo Scientific) and a pipette tip is used to create a flat, circular bed. The hypothalamic slices are gently pipetted onto the bed when set and residual L-15 is carefully removed. 40μl of collagen solution is pipetted on to the hypothalamic slice, and allowed to set for 30 minutes. When solid, 500μl of equilibrated slice culture media is added to each well. The collagen supports integrity of the tissue in three dimensions and allows sufficient diffusion of factors in culture media.

### 2.5.2 Culturing hypothalamic slices

Prior to optimisation of culture media, initial slice cultures were placed in neurosphere media. The final slice culture media comprises DMEM/F-12 (21331; GIBCO) supplemented with penicillin/streptomycin (15140122: GIBCO), 5μg/ml heparin (H3149; Sigma), L-Glutamine (25030024; GIBCO), B27 minus insulin (0050129SA; Life Technologies) and customised N2 lacking IGF and insulin. As evidence supports an IGF-1 induced proliferative response in tanycytes (Perez-Martin *et al.,* 2010), media lacking insulin and IGF are used in order to minimise proliferation in control cultures. Slices are cultured at 37°C and 5% $CO_2$ for a 24-hour recovery period, after which, experimental factors can be added to fresh media. Fresh media is provided every 24-hours in culture. Factors added to the media include FGF2 (13256029; Life Technologies), FGF10 (PHG0204; Life Technologies), FGF18 (PHG0234; Life Technologies), SU5402 (572631; Calbiochem), NMDA (M3262; Sigma), Kainate (ab120100; Abcam) and Dexamethasone (ab120743, Abcam). Dimethyl suplhoxide (DMSO, D2650; Sigma) was added to media where appropriate.

**Table 2.4: Slice culture standard regime**

| 0-24 hours | 24-48 hours | 48 hours |
|---|---|---|
| Recovery period | Experimental period | Fixation/processing |

**Table 2.5: Exogenous experimental factors in slice cultures**

| Biochemical | Working concentration | Source |
|---|---|---|
| BrdU | 10μM | Sigma |
| Dexamethasone | 10-100μM | Abcam |
| FGF10 | 60ng/ml | Life Technologies |
| FGF18 | 60ng/ml | Life Technologies |
| FGF2 | 60ng/ml | Life Technologies |
| KA | 50-100μM | Abcam |
| NMDA | 50-100μM | Sigma |
| SU5402 | 20μM | Calbiochem |

### 2.5.3 Processing and analysing hypothalamic slices

After the culture period, forceps are used to gently remove collagen from the slice. The hypothalamic slices are then processed for immunohistochemical techniques as stated in 2.2. To take fluorescent microscope images of hypothalamic slices, electrical tape is used to create thin wells on superfrost slides. The slice is pipetted into the well and excess solution is removed and replaced with Vectashield mounting media and a coverslip is placed on top. The slice is imaged using the AxioImager.Z1 with Apotome (Zeiss), and z-stacks of 70-100 1$\mu$m sections are compiled as a maximum intensity projection (MIP) using Axiovision software.

# Chapter 3

## Alpha-tanycytes are label-retaining, embryonically specified radial glial-like cells that express neural progenitor markers.

## 3.1 Introduction

Adult neurogenesis is strictly regulated within a specialised niche that dictates self-renewal and fate-determination. Factors involved in embryonic neurogenesis are largely responsible for maintaining proliferation and cell-specification in the adult niches, as described in detail above (see sections 1.1.2; 1.1.3). For this reason, characterisation of the factors that are expressed in the embryonic VZ of the hypothalamic 3rd ventricle provide an invaluable tool to assess the progenitor status of cells within the adult VZ. Furthermore, research supports an embryonic radial glial identity of adult neural stem cells in the SVZ of the lateral ventricles and the SGZ of the dentate gyrus. I therefore wanted to investigate the developmental origin of hypothalamic cells in order to elucidate the nature of the neural stem cell compartment in comparison to other adult sites. In this chapter, the adult tuberal hypothalamus will be interrogated alongside the corresponding embryonic region with antibodies against protein markers for neural progenitor status, and BrdU incorporation and retention will be analysed to further understand the origins of tanycytes. The E15 mouse is used in this study as embryonic neurogenesis reaches a peak at this stage (Shimada and Nakamura, 1973), making it a suitable model for neural progenitor characterisation, while the relatively late gestation stage provides a recognisable morphology relative to the adult and an opportunity to investigate the fate of VZ cells. Initially, photographs and illustrations will be used to provide orientation and context to subsequent experiments.

## 3.2.1 Orientation of the embryonic and adult hypothalamus

The hypothalamus is situated in the ventral diencephalon, bordered by the optic nerves laterally and optic chiasm rostrally, while the hypophysis (pituitary gland) serves as a caudal landmark (figure 3.1A). The hypothalamus consists of three major anatomical regions along the rostral-caudal axis: anterior, tuberal and mammillary. Tanycytes are a cell type found adjacent to the 3rd ventricle in the tuberal hypothalamus, broadly distinguished by the median eminence protrusion (light-blue oval). The median eminence is the ventral border of the 3rd ventricle and contains fenestrated capillaries of the portal blood system that connects it to the adenohypophysis (anterior pituitary) caudally.

Taking a slice through the tuberal hypothalamus (red line) provides a coronal cross-section illustrating the dorso-ventral axis in detail (figure 3.1B). The illustration demonstrates that the embryonic hypothalamus at E15 is morphologically similar to the adult; however expansion in size occurs around the 3$^{rd}$ ventricle and terminal differentiation of cell types within nuclei ensures a physiologically functional hypothalamus at birth, although development continues postnatally. The coronal illustration focuses on the ventral diencephalon, the region that will be investigated in this thesis, while the other major diencephalic structure, the thalamus, is situated dorsally. Notably, surrounding the E15 3$^{rd}$ ventricle, a distinct VZ can be observed that is many cells thick; in adult mice this region is substantially leaner. As indicated in the illustration at E15, neurogenesis is occurring, but terminal differentiation and agglomeration of neurons into defined nuclei is not complete. Conversely, clear nuclei are observable in the adult, each comprised of numerous neuronal cell types. Of particular importance to this thesis, the tanycyte subtypes that encompass the ventral VZ of the adult are shown in their relative locations (see figure 3.1B legend).

Considering the gross morphological similarities between the embryonic and the adult hypothalamus, and the recent evidence in support of a native neural stem cell population, potentially in the alpha-tanycyte territory, the aim of this chapter is to determine the developmental origin of alpha-tanycytes, and to identify whether, as in the SVZ and SGZ, hypothalamic tanycytes share features of the embryonic radial glia.

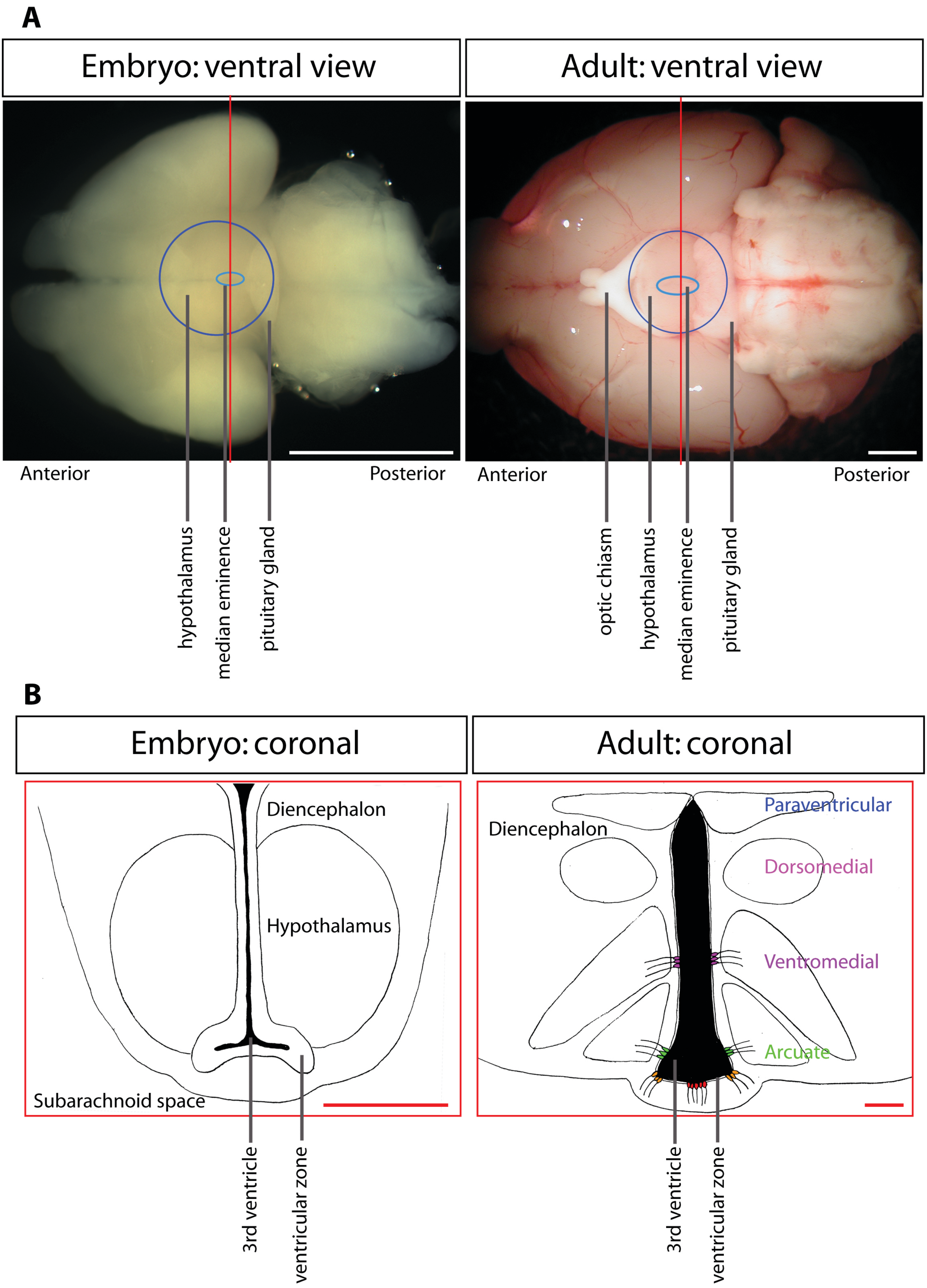
### 3.2.2 Embryonically expressed neural progenitor markers are maintained in adulthood

Antibodies against proteins, established as embryonic neural progenitor markers, were used to investigate the progenitor status of cells within the

**Figure 3.1: Identification and orientation of the hypothalamic 3rd ventricle in the embryonic and adult brain.**

A. Photographs of the ventral side of the embryonic and adult brain. The optic chiasm of the optical nerves (CNII) is a rostral landmark. Dark-blue circles signify the boundary of the hypothalamus at both embryonic day (E) 15, and postnatal day (P) 35. Light-blue ovals indicate the position of the median eminence, the ventral border of the 3rd ventricle, anterior to the pituitary gland. Scale bar represents 2mm.

B. Illustrations show coronal views at the level of the red line at both E15 and P35. At E15 the ventricular zone (VZ) is thick. At P35, the VZ is apparent, but has reduced thickness. Tanycyte subpopulations are shown within the P35 VZ; hypothalamic nuclei that border the 3rd ventricle at the level of the median eminence (tuberal hypothalamus) are outlined. Tanycyte subpopulations are coloured accordingly: alpha-1, purple; alpha-2, green; beta-1, orange; beta-2, red. Scale bar represents 100$\mu$m


A
Embryo: ventral view
Adult: ventral view
Anterior
Posterior
Anterior
Posterior
hypothalamus
median eminence
pituitary gland
optic chiasm
hypothalamus
median eminence
pituitary gland
B
Embryo: coronal
Adult: coronal
Diencephalon
Hypothalamus
Subarachnoid space
3rd ventricle
ventricular zone
Diencephalon
Paraventricular
Dorsomedial
Ventromedial
Arcuate
3rd ventricle
ventricular zone

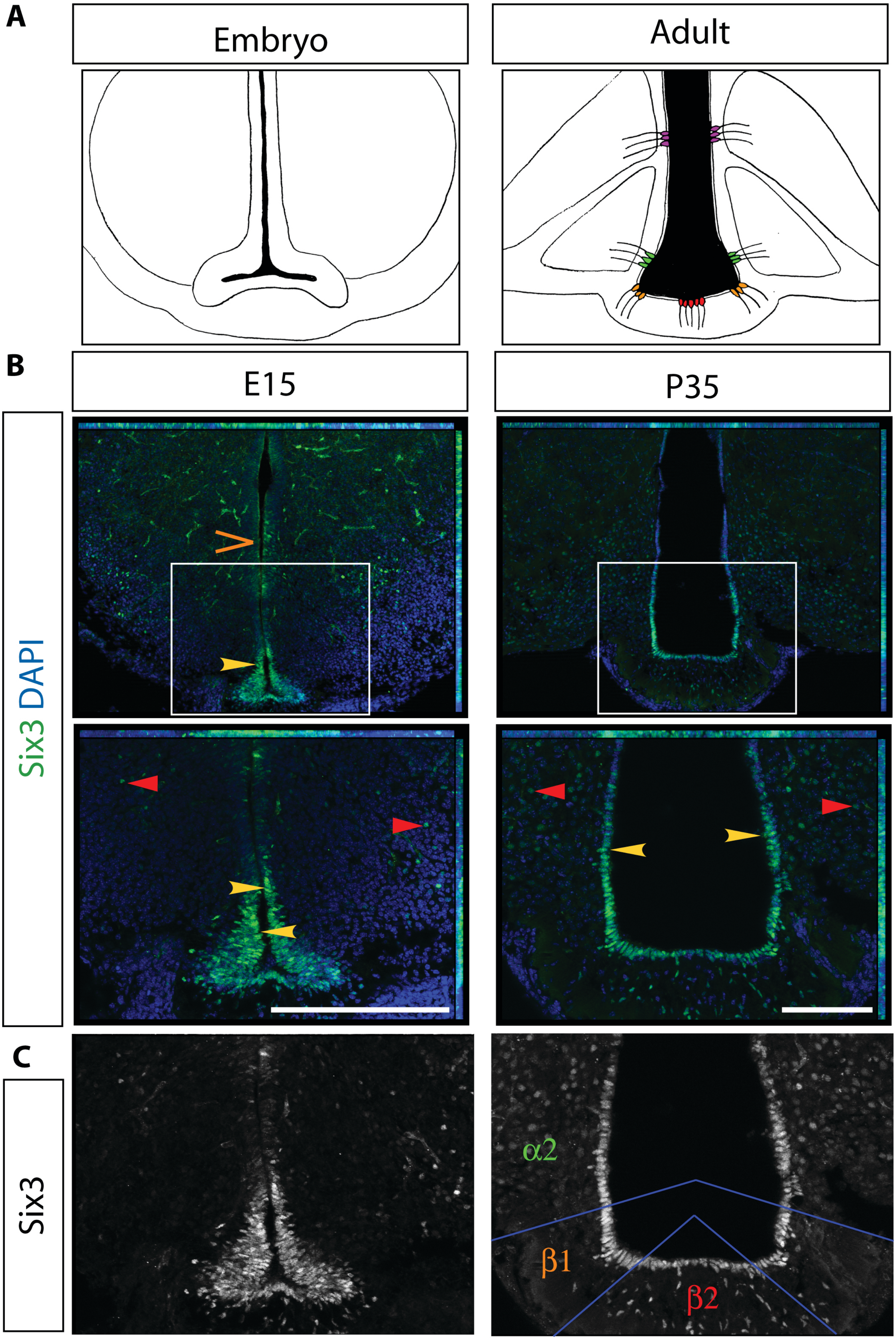
tuberal hypothalamus in order to identify cells of a potential adult neurogenic niche.

Evidence supports *Six3*, a homeobox protein crucial for the development of the anterior neural plate and forebrain, in controlling the fine balance of proliferation and differentiation (Appolloni *et al.*, 2008). Adjacent to the embryonic 3rd ventricle, Six3 is strongly expressed in the ventral (yellow arrowheads) and medial VZ (orange arrowhead) (figure 3.2B), with weaker expression dorsally, and a weak region of expression between the ventral and medial sites. This embryonic expression pattern, localised primarily to the VZ, supports Six3 as a marker of neural progenitor status. A similar expression pattern of Six3 is apparent in the adult VZ, with high levels in the region of alpha2- (yellow arrowheads) and beta1-tanycytes and a weaker expression in dorsal alpha-tanycytes and medial beta2-tanycytes (figure 3.2B,C). At both embryonic and adult stages, Six3 positive cells can be observed in the hypothalamic parenchyma (red arrowheads) supporting either an additional neural progenitor population present in the parenchyma or a role in terminal differentiation.

Sox3, a member of the SoxB1 family of transcription factors, is required for the correct development of the central nervous system, and expression is maintained in neural progenitor cells of the SGZ and SVZ where it inhibits differentiation (Rogers *et al.*, 2013). At E15, Sox3 is widely expressed in the VZ along the dorso-ventral axis of the 3rd ventricle, supporting its role in maintaining an undifferentiated state in neural progenitors (figure 3.3B). By adulthood, expression is restricted to the ventral VZ, corresponding to the location of both alpha- and beta-tanycytes, supporting an undifferentiated state of cells within this region (figure 3.3C). In addition, Sox3-positive cells are distributed throughout the embryonic parenchyma and the adult arcuate and ventromedial nuclei, suggesting a role of Sox3 in mature hypothalamic cell types in addition to their function in neural progenitors. In accordance with this, evidence suggests an independent role for Sox3 in mature cell types, within a number of hypothalamic nuclei (Rogers *et al.*, 2013). Although Sox3 expression is not limited to neural progenitors within the hypothalamus, the localised expression

**Figure 3.2: Homeobox protein, Six3, is expressed in the VZ of the embryonic and adult tuberal hypothalamus.**

A. Illustrations show orientation for coronal plane images in B
B. Maximum Intensity Projections (MIPs) of E15 and P35 tuberal hypothalamus are shown, immunohistochemically labelled for the nuclear marker, DAPI (blue), and the homeobox protein Six3 (green). Strong expression of Six3 is observed in the ventral VZ (yellow arrowhead) and medial VZ (orange arrowhead) of the 3rd ventricle in the E15 tuberal hypothalamus; at P35 strong expression is present in the ventral VZ. Magnifications of the boxed regions are shown. Yellow arrowheads point to strong expression in the VZ, in the alpha-tanycyte region. Expression of Six3 is also detected in parenchymal cells in both the embryo and adult tuberal hypothalamus (red arrowheads). Scale bar represents 100$\mu$m.
C. MIPs of E15 and P35 tuberal hypothalamus immunohistochemically labelled for Six3. P35 image is annotated to define tanycyte subtype region.

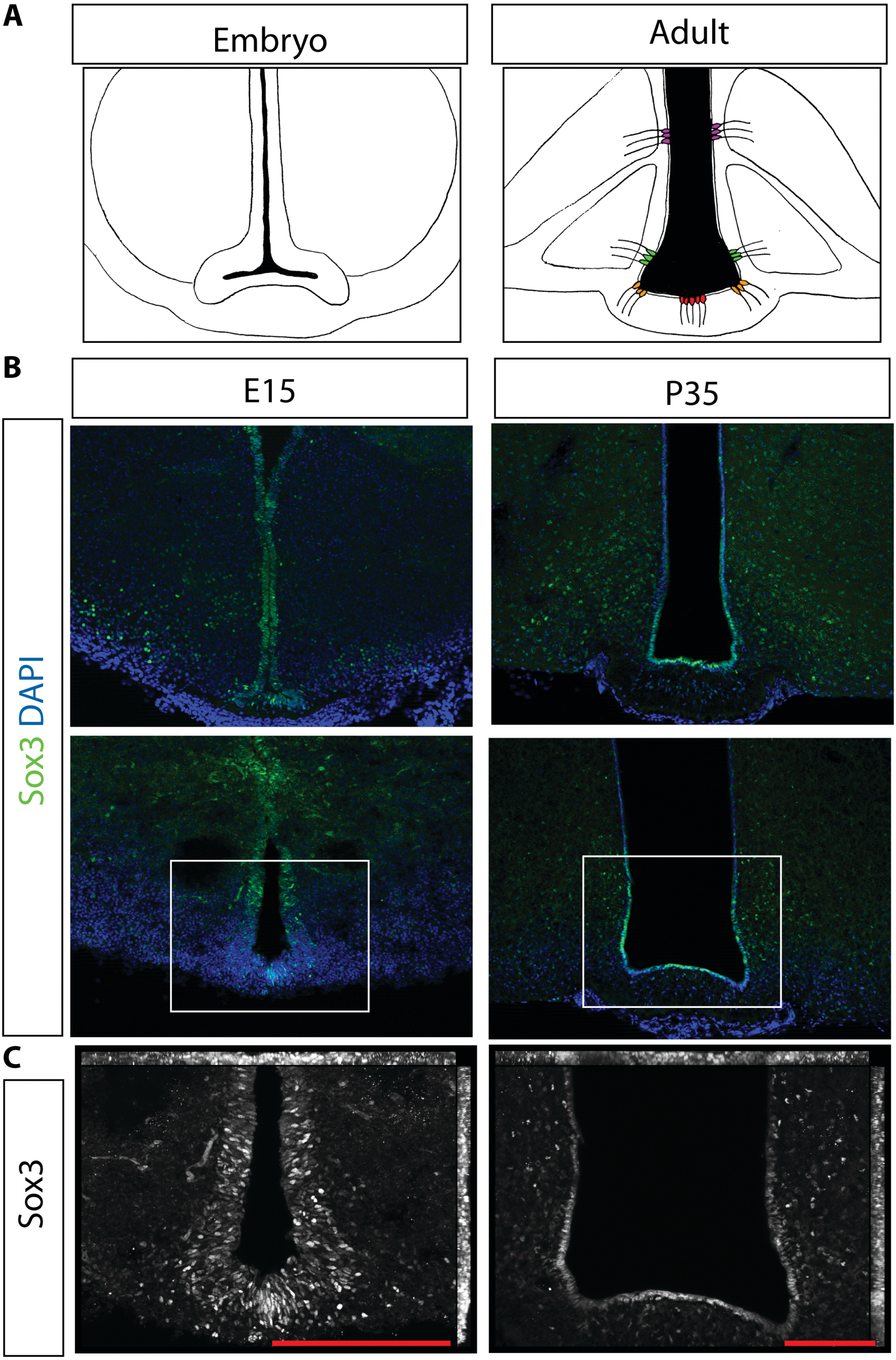

A
Embryo
Adult
B
E15
P35
Six3 DAPI
C
Six3
α2
β1
β2

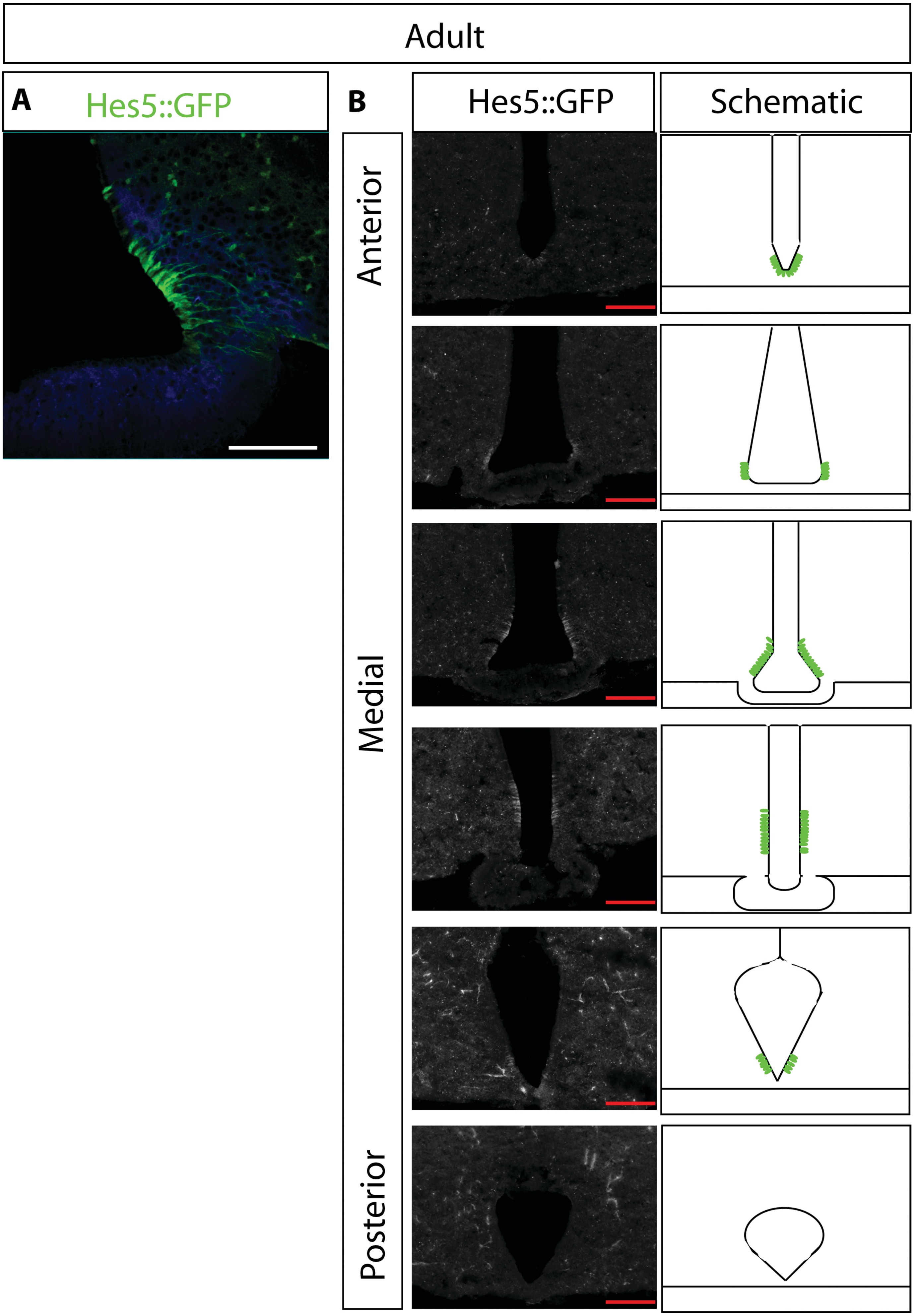
## Figure 3.3: Embryonic transcription factor, Sox3, is expressed in the VZ of the embryonic and adult tuberal hypothalamus.

A. Illustrations show orientation for coronal plane images in B
B. Immunofluorescence images are shown labelled with nuclear marker, DAPI (blue), and hypothalamic neural progenitor marker, anti-Sox3 (green). Sox3 is widely expressed along the entire dorso-ventral axis of the VZ at E15. At P35 expression in the VZ is strongest ventrally, the region rich in tanycytes.
C. Boxed regions in B are magnified and shown as MIPs immunohistochemically stained for Sox3. Scale bar represents 100μm.

## Figure 3.4: Expression of Notch signalling effector, Hes5, is restricted to alpha2-tanycytes in the adult VZ

A. Immunofluorescent image of Hes5::GFP mouse tuberal hypothalamus, hes5::GFP is shown in green. Expression is restricted to alpha2-tanycyte subtype. Scale bar represents 100μm
B. Immunofluorescent images of Hes5::GFP mouse hypothalamus at six different levels through the 3rd ventricle anteroposteriorly. A schematic of the staining is shown adjacently. Hes5::GFP shows restricted expression to a subset of VZ cells along the anteroposterior axis. Scale bar represents 100μm.


A
Embryo
Adult
B
E15
P35
Sox3 DAPI
C
Sox3


Adult
A
Hes5::GFP
B
Hes5::GFP
Schematic
Anterior
Medial
Posterior

in the VZ of the ventral $3^{rd}$ ventricle, along with other neural progenitor markers, suggest it is a marker of neural progenitor identity here.

Hes5 is a member of the Hes family of Notch effectors, expressed by neural progenitors in the mouse embryo, where it acts as a transcriptional repressor of proneural genes (Cau *et al.*, 2000). Previous studies have shown the transgenic mouse reporter-line for notch effector, Hes5 (*Hes5::GFP*), specifically marks neural stem cells within the subgranular zone of the dentate gyrus (section 1.1.3, page 34) (Lugert *et al.*, 2010). The same reporter-line is analysed for expression in the adult tuberal hypothalamus. GFP expression shows an extremely specific expression pattern, restricted to alpha2-tanycytes (figure 3.4A). When the anteroposterior axis of the $3^{rd}$ ventricle is analysed for GFP expression, a regional discrepancy is observed at each level, as the position of GFP-positive cells relative to the ventricle changes (figure 3.4B). These data support the presence of a neural progenitor within the alpha2-tanycyte subpopulation, and suggest tanycyte subtype distribution changes in parallel with the morphological variance along the anteroposterior axis of the adult $3^{rd}$ ventricle.

Radial glial cells are the neural progenitor population during embryogenesis (section 1.1.5), and can be specifically identified using the RC2 antigen, encoded by *Nestin* (Park *et al.*, 2009). At E15, RC2 is widely expressed by cells with a radial glial morphology along the dorso-ventral axis of the $3^{rd}$ ventricle, supporting their central role as neural precursors during development of the hypothalamus (figure 3.5B). By adulthood, as defined by sexual maturity, expression of the embryonic radial glial antigen, RC2, is restricted to cell types in the ventral region of the $3^{rd}$ ventricle, the region rich in tanycyte cell-types. In the adult, anti-RC2 labels cells morphologically similar to embryonic radial glia, with a cell body in the ventricular zone (VZ) and a basal projection. Based on the location and direction of the labelled projections towards the arcuate nucleus, it is evident that alpha2-tanycytes express radial glia cell marker (yellow arrowheads). Similarly the lateral population of beta1-tanycytes are positive for the RC2 protein (red arrowheads), while expression appears to decrease medially (figure 3.5C).

A further distinguishing feature of embryonic radial glial cells is the presence of a single primary cilium on the apical surface, extending into the ventricle. The small GTPase, Arl13b, is associated with microtubule regulation in primary cilia, required for the intraflagellar transport of Shh signalling components (Larkins *et al.*, 2011; Higginbotham *et al.*, 2013). Arl13b is therefore a useful marker of primary cilia, and is used here to distinguish potential progenitors from terminally differentiated, multi-ciliated ependymal cells in the hypothalamic VZ. At E15 Arl13b-positive protrusions are observed along the dorsoventral axis of the 3$^{rd}$ ventricle, and are maintained into adulthood (figure 3.6B). Analysing alpha-tanycyte regions compared to beta-tanycyte regions reveals abundant primary cilia in beta1-tanycyte regions and dorsally, while sparse numbers of primary cilia are observed in the beta2-tanycyte region (figure 3.6C). High-power images of the VZ confirm the primary cilium morphology of Arl13b-positive tissue.

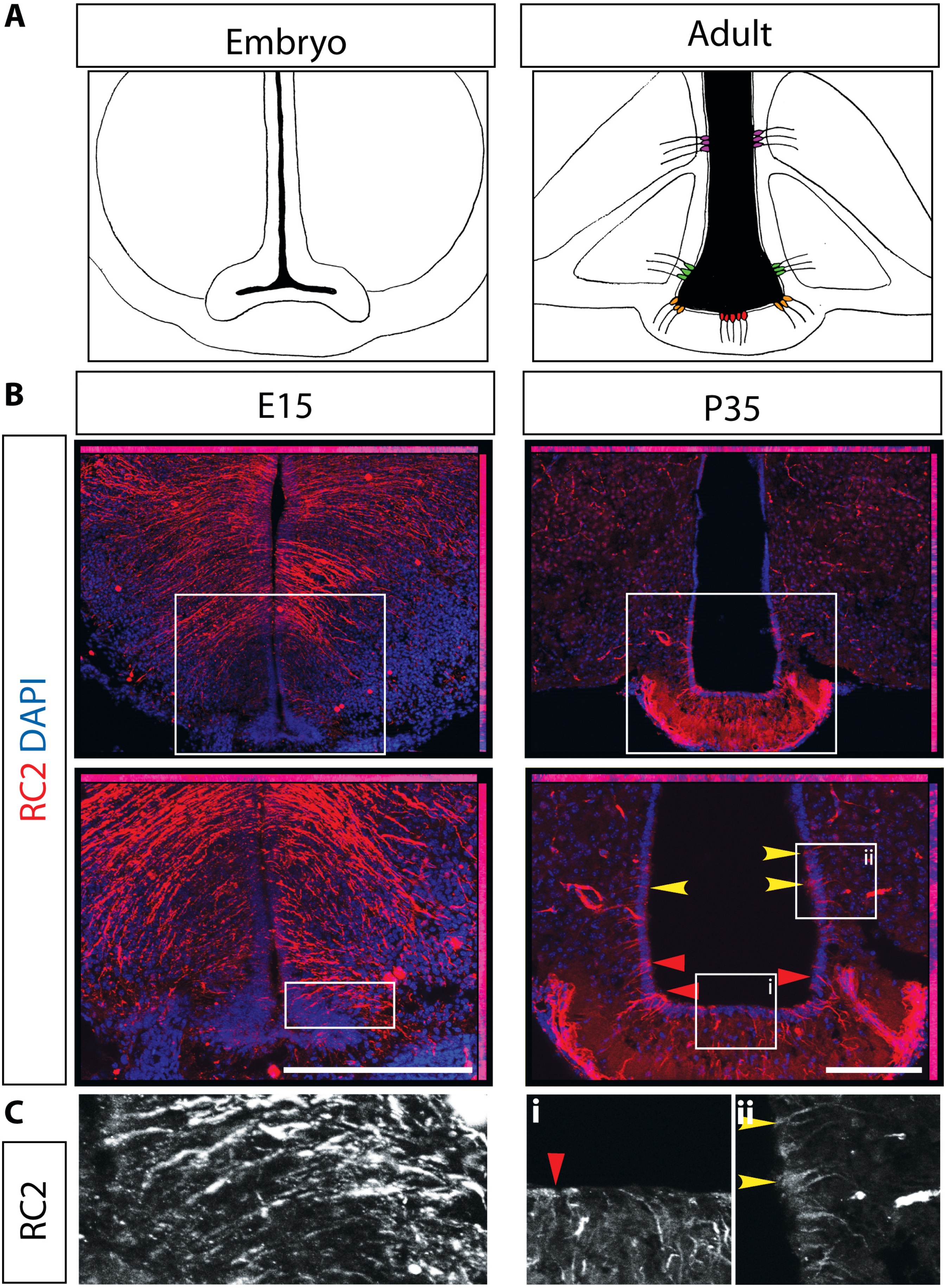
Taken together these results support the presence of neural progenitors within the adult hypothalamic VZ, that maintain expression of embryonic radial glia and neural stem/progenitor proteins including Six3, Sox3 and Hes5, all of which are involved in self-renewal and preventing terminal differentiation. Interestingly, tanycyte subtypes also express the intermediate filament marker, RC2, in their basal projections, and the primary cilia marker Arl13b on their apical surface, both of which are characteristic features of radial glia in the embryonic neuroepithelium. These data provide evidence that within the alpha- and beta-tanycytes of the adult hypothalamus, sub-population(s) share neural progenitor characteristics with the embryonic ventricular zone.

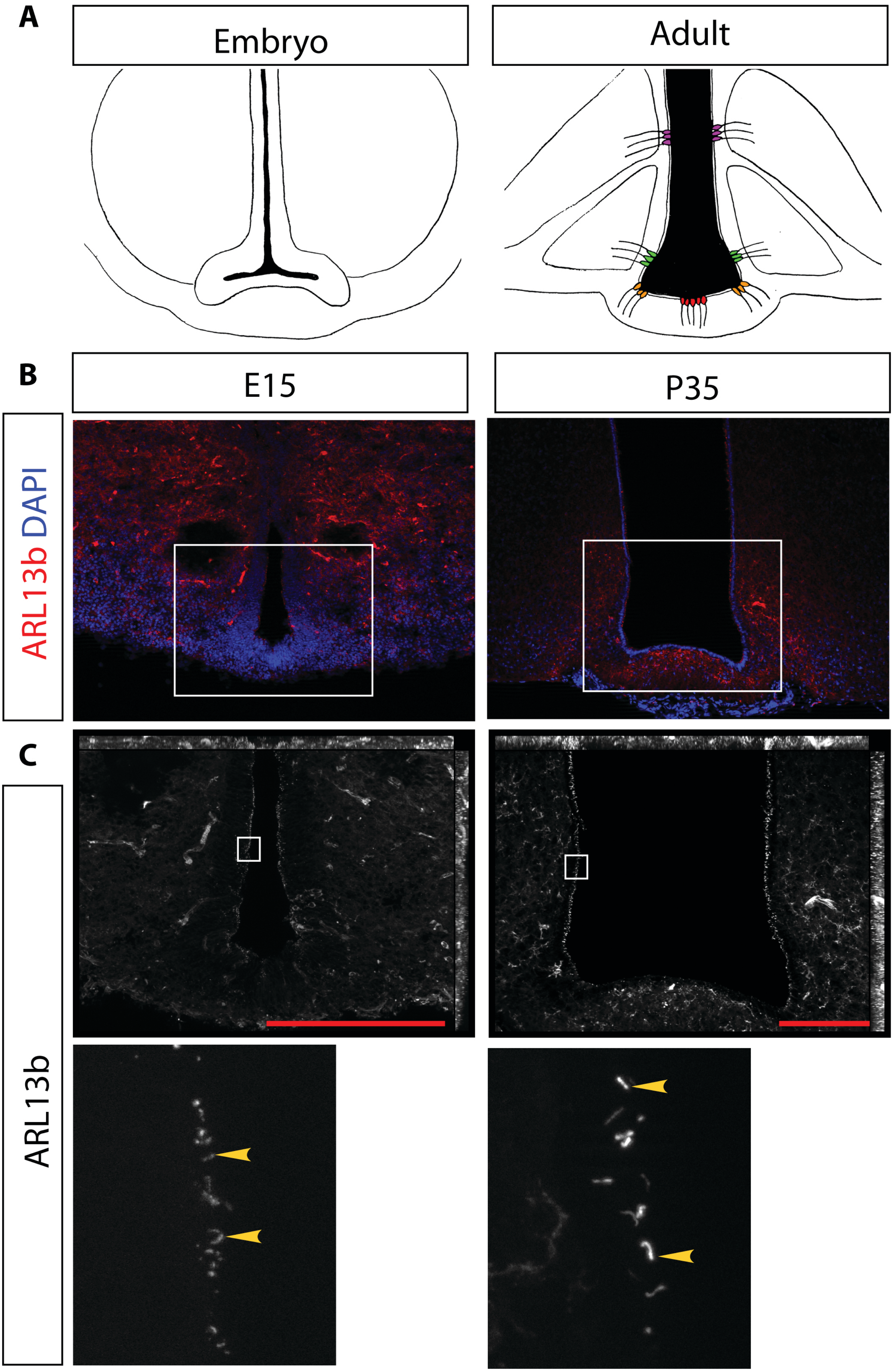
## Figure 3.5: Radial glial cell marker, RC2, is expressed in the VZ of the embryonic and adult tuberal hypothalamus.

A. Illustrations show orientation for coronal plane images in B
B. MIPs of E15 and P35 tuberal hypothalamus, labeled with nuclear marker, DAPI (blue), and radial glial cell marker, anti-RC2 (green). RC2 is widely expressed along the dorso-ventral axis at E15. At P35, RC2 expression is restricted to cell types in the ventral portion of the 3rd ventricle VZ. Magnifications of the boxed ventral 3rd ventricle are shown. Yellow arrowheads indicate expression of RC2 in cells morphologically identical to alpha-tanycytes in the P35 tuberal hypothalamus. Red arrowheads indicate expression of RC2 in cells morphologically identical to beta1-tanycytes. Scale bar represents 100$\mu$m.
C. Magnifications of boxed regions in B, showing RC2 antigen expression. RC2-positive projections are shown in embryonic VZ. In the adult, red arrowheads show expression in beta-1 tanycytes (i); yellow arrowheads show expression in alpha-2 tanycytes (ii).

## Figure 3.6: Hypothalamic ependymal cells display Arl13b-positive primary cilia

A. Illustrations show orientation for coronal plane images in B
B. Fluorescent images of the E15 and P35 tuberal hypothalamus are shown, labelled with nuclear marker, DAPI (blue); and the small GTPase enriched in primary cilia, Arl13b (red). ARL13b-positive signal can be seen lining the 3rd ventricle in both the embryo and adult.
C. MIPs for Arl13b are shown as magnifications of the boxed regions in B. Strong signal is detected lining the 3rd ventricle of the embryo and adult. In the adult expression is abundant at the level of alpha-tanycytes and sparse at the level of beta-tanycytes. Magnifications of the boxed regions show morphology of primary cilia at both E15 and P35. Scale bar represents 100$\mu$m.


A
Embryo
Adult
B
E15
P35
RC2 DAPI
ii
i
C
RC2
i
ii


A
Embryo
Adult
B
E15
P35
ARL13b DAPI
C
ARL13b

### 3.2.3 BrdU retention reveals a subpopulation of tanycytes are slow-dividing progenitors

The data above support a radial glial identity of subpopulation(s) of tanycytes within the ventral VZ of the tuberal hypothalamus. In order to determine whether cells in this region are an adult cell type that has gained radial glia characteristics, or an embryonically specified cell type that is maintained into adulthood, BrdU incorporation was used as a reporter of cells that had undergone S phase of the cell cycle and subsequent mitosis.

Gross embryonic neurogenesis peaks at E14 and E15, therefore BrdU was injected into a pregnant dam at embryonic day 14, 15, 16 and 17 to capture the dynamics of proliferation in the hypothalamus around the established peak of embryonic neurogenesis (figure 3.7A). Twelve-hours after the last BrdU injection and twenty four-hours after the first, the embryos were fixed and analysed in order to obtain an account of the proliferation during each embryonic day studied. Antibody labelling against BrdU reveals cells at the VZ of the 3$^{rd}$ ventricle had progressed through the cell cycle at a varying rate during the different embryonic ages (figure 3.7B). Quantifying the number of VZ cells that have incorporated BrdU shows that, as in other regions of the central nervous system, proliferation is high at E14 and peaks at E15 (figure 3.7C). Importantly, only cells observed to make contact with the ventricle are quantified in order to provide a comparable standard for the different ages. After E15 the number of VZ cells that undergo proliferation during the injection regime significantly decreases from a mean of 52 (±2.8) cells per section to 10 (±1.6) at E17. As the analysis was performed twenty-four hours after the first injection, the mitosis marker phosphorylated-histone 3 (PH3) was used to quantify the number of cells undergoing proliferation at the time of fixation. Quantification of PH3-positive VZ cells supports the BrdU analysis, showing a peak of proliferation at E16 with a mean of 15.4 (±1.5) cells per section, and 1.5 (±0.6) cells at E18 (figure 3.7D). These data suggest that the majority of 3$^{rd}$ ventricle VZ cells undergo their last round of division during E15 and E16, or alternatively, become slow-dividing cells at this stage.

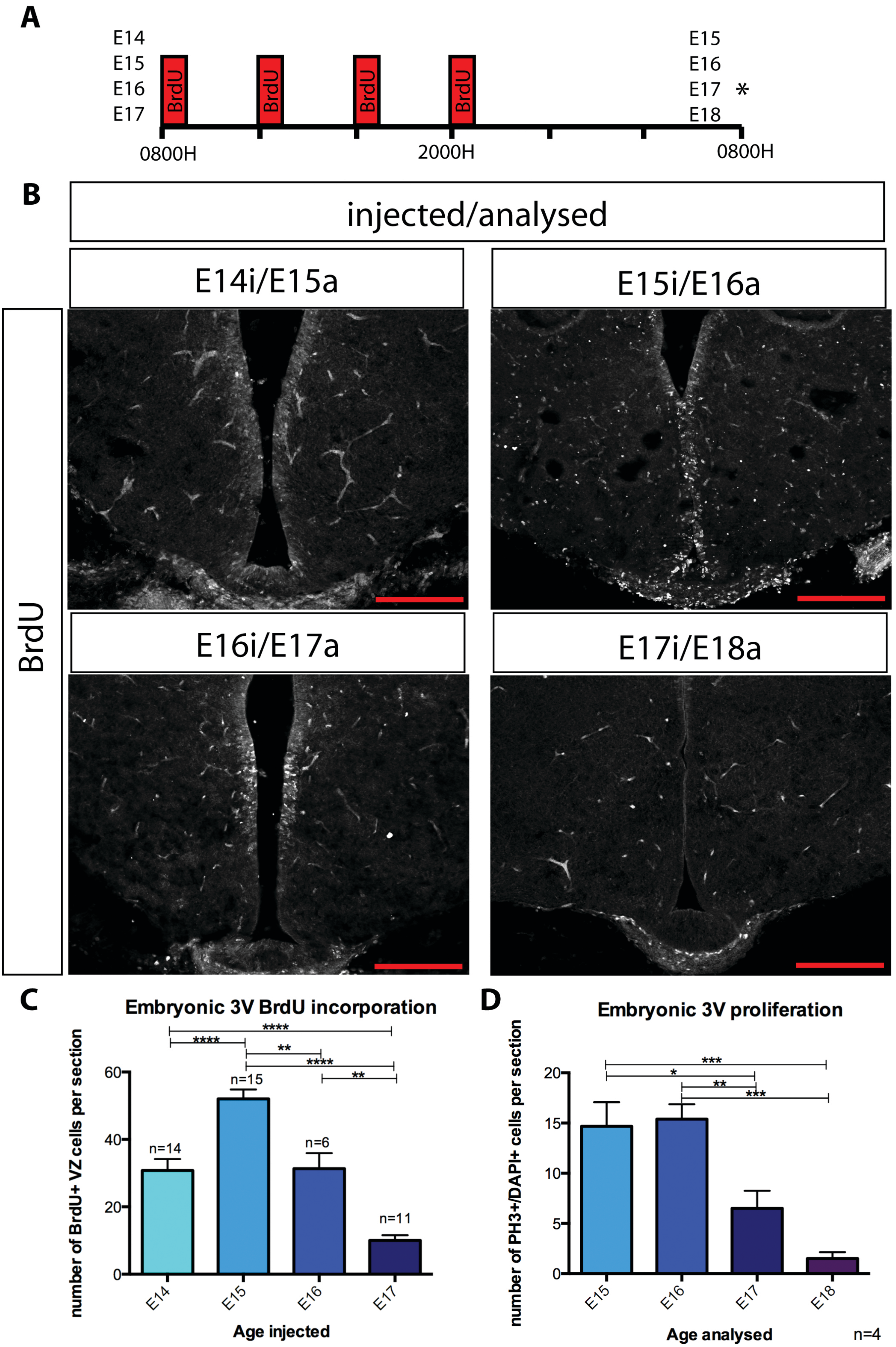
## Figure 3.7: Proliferation in the VZ of the tuberal hypothalamus is at a peak at E15 and E16.

A. Schematic representation of BrdU injection regime: pregnant mice with embryos at stages E14, E15, E16, E17, were injected with BrdU (100mg/kg) four times in 16 hrs, at four-hour intervals. The mother was sacrificed twelve-hours after the last injection (represented with large asterisk) and the embryos were processed for BrdU incorporation.

B. Immunofluorescence images of BrdU, after injection at different embryonic ages. Incorporation of BrdU during cell cycle appears strongest and most widespread dorso-ventrally during the E15 injection regime, with incorporation still strong during the E16 regime. Incorporation appears weaker during the E14 injection regime, and BrdU incorporation is almost absent at the VZ by E17. Scale bar represents 100$\mu$m.

C. Quantification of the number of BrdU positive cells in contact with the ventricle (VZ) per 20$\mu$m section (E14, $n=14$; E15, $n=15$; E16, $n=6$; E17, $n=11$) shows a peak at E15 with significantly more incorporation than during the other injection dates. ($p<0.05$ = *)

D. Quantification of sections ($n=4$) immunohistochemically labelled for phosphorylated-histone3 (PH3) shows a significant decrease in the number of dividing cells at the time of sacrifice with increasing embryonic age ($p<0.05$ = *).


A
E14
E15
E16
E17
BrdU
BrdU
BrdU
BrdU
E15
E16
E17 *
E18
0800H
2000H
0800H
B
injected/analysed
E14i/E15a
E15i/E16a
E16i/E17a
E17i/E18a
BrdU
C
Embryonic 3V BrdU incorporation
number of BrdU+ VZ cells per section
0
20
40
60
n=14
n=15
n=6
n=11
****
****
**
****
**
E14
E15
E16
E17
Age injected
D
Embryonic 3V proliferation
number of PH3+/DAPI+ cells per section
0
5
10
15
20
***
*
**
***
E15
E16
E17
E18
Age analysed
n=4

Retention of BrdU in the DNA of cells, a period of time after its incorporation, is a classical assay used to detect quiescent haematopoietic stem cells (Challen *et al.*, 2009). The detection of the BrdU-label suggests that either the cell has undergone terminal differentiation or that the cell has entered a quiescent state. Multiple rounds of division will lead to a dilution of the amount of BrdU as new DNA is synthesised and distributed to the daughter cells. BrdU-retention is therefore frequently used to elucidate the stem cell characteristics of different populations. As the previous data indicated a peak of proliferation at E15 and E16, followed by a significant decrease, if the BrdU incorporation at this peak was retained in the VZ of the adult hypothalamus then it would support an embryonic specification and a slow-dividing nature of an adult neural stem cell population. To address this, BrdU was injected into a pregnant dam four times per day for two days starting at E15, the pups were then sacrificed at different life-stages to subsequently analyse the amount of BrdU retention (figure 3.8A).

Analysis of labelling at birth, before and after sexual maturity and in the fully matured adult, shows BrdU is retained at all ages investigated (figure 3.8B). The retention pattern in the P35 and P49 adult are of particular interest, showing a high level of BrdU in the VZ of the alpha-tanycyte region (yellow arrowheads), and a weaker (although not absent) level of BrdU in the beta-tanycyte region as well as dorsal VZ (red arrowheads). The quantification of VZ BrdU-labelling at the different ages reveals an increase in the number of BrdU from a mean of 64.5 (±3.2) at P0 to 200.5 (±8.8) at P14 (figure 3.8C), suggesting a postnatal and pre-pubertal proliferation that generates BrdU-positive daughters that remain in the VZ. However, due to the differences in hypothalamic size between P0 and P14, a direct statistical comparison would be inappropriate and is not made. At the adult ages of P35 and P49, a lower number of VZ BrdU-positive cells are observed at 107 (±10.1) and 154.1 (±7.6) respectively. Interestingly, a difference in the number of BrdU-positive cells can be seen between males and females at P14 and P35 (figure 3.8D) suggesting a sexual dimorphism in proliferation during puberty. This is further identified using a graphical representation of the number of VZ BrdU-positive cells along the anterior-posterior axis at P0 and P35 (figure 3.8E,F), showing comparable numbers of VZ BrdU-positive cells in the P35 male and the P0 pups. This

## Figure 3.8: Embryonically incorporated BrdU is retained in the VZ of the adult tuberal hypothalamus

A. Schematic, showing the BrdU injection regime. BrdU was injected (100mg/kg) four times per day on E15 and E16, at four-hour intervals. Siblings were then sacrificed at parturition, postnatal day 0, or at the pre-pubertal date P14, the sexually mature date P35, or as fully matured adults at P49. Large asterisks indicate the day of sacrifice.

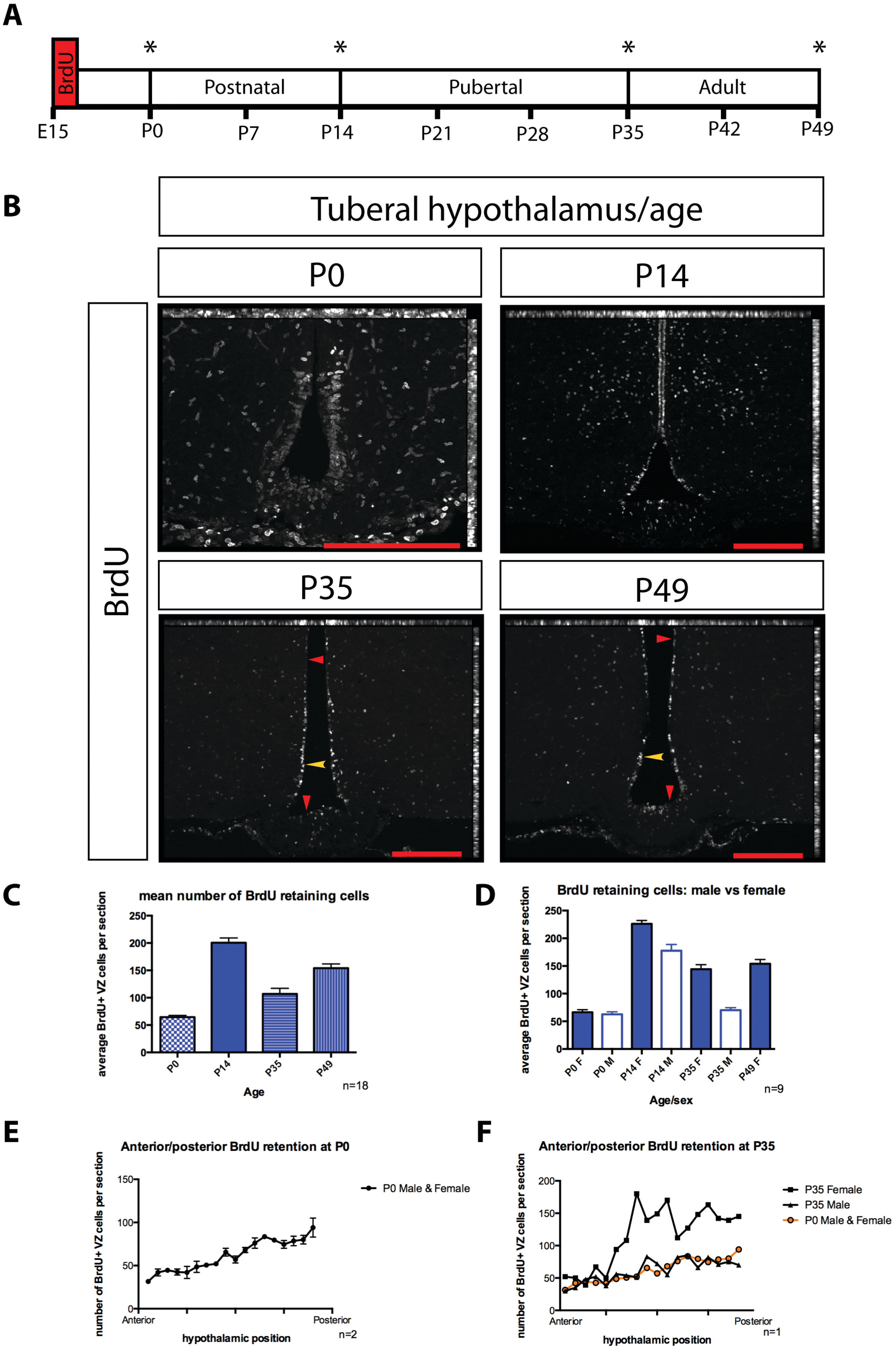
B. Immunofluorescence MIPs are shown of embryos subject to BrdU incorporation during E15/E16 injection regime, and sacrificed at P0, P14, P35 and P49. Strong BrdU retention is observed in the VZ across all ages into adulthood, with a particularly high retention level in the alpha-tanycyte region (yellow arrowhead) and a relatively lower retention rate in the beta-tanycyte region and dorsal ependyma (red arrowhead) at P35/P49. Scale bar represents 200$\mu$m.

C. Nine tuberal sections from two siblings, male and female, at each age were quantified for VZ BrdU retention, the average of which is shown in C (*n=18*). Due to the morphological changes in size that occur at the 3rd ventricle from P0 through to adult, a direct statistical comparison of BrdU retention is not made; however, the graph shows an increase in VZ BrdU at the onset of puberty, which declines by sexual maturity and adulthood.

D. Presenting the data according to gender (*n=9*) reveals the amount of BrdU retention is similar at P0, and increases at the onset puberty, although a significant amount of sexual dimorphism is apparent in the amount of retention at P14 and P35.

E. Shows the graphical representation of the number of BrdU retaining cells along the anterior-posterior axis of the 3rd ventricle, at P0 (*n=2*). A trend reveals an increase in the amount of BrdU in tuberal and posterior regions.

F. A similar trend is observed at P35 (*n=1*), with a high level of BrdU retention in tuberal and posterior regions in females, and in males the amount of BrdU retention along the anterior-posterior axis is comparable to P0 mice.


A
BrdU
*
Postnatal
Pubertal
Adult
E15
P0
P7
P14
P21
P28
P35
P42
P49
B
Tuberal hypothalamus/age
P0
P14
P35
P49
BrdU
C
mean number of BrdU retaining cells
average BrdU+ VZ cells per section
0
50
100
150
200
250
P0
P14
P35
P49
Age
n=18
D
BrdU retaining cells: male vs female
average BrdU+ VZ cells per section
0
50
100
150
200
250
P0 F
P0 M
P14 F
P14 M
P35 F
P35 M
P49 F
Age/sex
n=9
E
Anterior/posterior BrdU retention at P0
number of BrdU+ VZ cells per section
0
50
100
150
Anterior
Posterior
hypothalamic position
n=2
P0 Male & Female
F
Anterior/posterior BrdU retention at P35
number of BrdU+ VZ cells per section
0
50
100
150
200
Anterior
Posterior
hypothalamic position
n=1
P35 Female
P35 Male
P0 Male & Female

suggests that the number BrdU-positive cells at P0 represents the basal level of retention, and thus a population that generates daughter cells prior to P14, which then migrate or die before analysis of the P35 male. Furthermore, the trend of an increase in BrdU-retention in the medial and posterior hypothalamic regions correlates well with the regions that are rich in tanycytes.

These BrdU data provide support to the notion that a subpopulation(s) of adult VZ cells, potentially tanycytes, are an embryonic cell type that enters a relatively quiescent state at E15/E16, preventing the label from being diluted through multiple rounds of division. Furthermore, the label is retained into adulthood where the highest level of retention can be observed in the region of the alpha-tanycyte population. Interestingly, a substantial amount of sexual difference is found in the amount of proliferation postnatally, and during puberty, suggesting both a role for progenitor cells during this period as well as a function related to sexual maturation.

## 3.3 Discussion

Although recent studies have begun to characterise hypothalamic tanycyte/ependymal cells on the basis of their expression of neural progenitor markers (Lee *et al.*, 2012a; Hajihosseini *et al.*, 2008; Xu *et al.*, 2005), these studies have not been exhaustive. The aim of this research was to compare expression patterns of established neural progenitor markers at a time known to be neurogenic, E15, with those of the relatively quiescent adult. Additionally, studies have not investigated the developmental origins of different hypothalamic tanycytes. Therefore a second goal was to determine whether tanycytes might be remnant radial glial cells from this region, or a cell-type specified elsewhere. In achieving these aims I hoped to gain a better understanding of progenitor status of tanycyte cells, and improve our current understanding of the relationship between tanycyte subtypes.

### 3.3.1 Alpha-tanycytes display neural progenitor features

My studies demonstrate expression of a number of progenitor markers within the VZ of the 3rd ventricle, specifically, expression of Six3, Sox3 and Hes5. All three are expressed within the alpha 2-tanycyte subset.

Expression of the homeodomain transcription factor Six3 is initiated early and exclusively in the anterior neural plate (Gestri *et al.*, 2005). Six3 is recognised as a crucial regulator of the development of anterior neural tissue in vertebrate embryos: *Six3*-null mouse embryos exhibit a severely truncated forebrain (Lagutin *et al.*, 2003). In humans, mutations in *Six3* result in holoprosencephaly (HPE), a malformation in which the forebrain fails to separate into two hemispheres and craniofacial defects occur including cyclopia (Carlin *et al.*, 2012). Hypomorphic mutations in Sonic hedgehog (Shh) are also an established cause of HPE, and studies have now provided evidence of an interaction between Six3 and Shh in the development of the forebrain, whereby they mutually regulate each others' transcription (Jeong *et al.*, 2008; Carlin *et al.*, 2012). Furthermore, Six3 promotes the proliferation of forebrain progenitors by antagonizing Geminin, a DNA replication inhibitor (del Bene *et al.*, 2004), and cells in the embryonic forebrain can be maintained in an undifferentiated state when a retroviral vector is used to upregulate *Six3* expression (Appolloni *et al.*, 2008). Considering the importance of Shh in the regulation of adult neurogenesis (sections 1.1.2, 1.1.3), and the finding that *Six3* mRNA is expressed in the classically defined neurogenic niches of the SVZ and SGZ postnatally (Appolloni *et al.*, 2008; Gray *et al.*, 2004), Six3 expression presents a useful tool to discern the progenitor status of adult VZ cells. The observable high expression levels in alpha2-tanycyte and beta1-tanycyte subtypes support a neural progenitor population within this region, while the varying levels of expression surrounding this region could be attributed to cells at different points of specification and differentiation (figure 3.2B). Although Six3 expression is not restricted to a single tanycyte-subtype, these data support a population(s) of neural progenitors in the ventral VZ of the 3rd ventricle.

The Sox family of transcription factors contain a highly conserved DNA binding domain and have important roles in self-renewal and differentiation. Recent evidence has elucidated the contribution of Sox3 to the development of the central nervous system: Sox3 inhibits neural differentiation in the diencephalon, its over-expression resulting in congenital hydrocephalus, a severe excess of CSF in the ventricles leading to increase intracranial pressure and chance of death (Lee *et al.*, 2012b). Studies investigating the expression patterns of Sox3 find it to be a reliable marker for neural progenitor cells throughout embryonic neurogenesis as well as in the adult SVZ and SGZ (Rogers *et al.*, 2013). Furthermore, recent evidence has identified ventral hypothalamic progenitors, shown to contribute to the developing infundibulum (future neurohypophysis), to be Sox3-positive in the chick (Pearson *et al.*, 2011). My analyses show that a ventral Sox3-positive cell population is maintained in the adult murine hypothalamic VZ, supporting an adult neural progenitor population(s) within alpha- and beta-tanycytes (figure 3.3B). Interestingly, Sox3 expression in the hypothalamus shows a characteristic seemingly unique to this region: it is found in both immature and mature neurons in addition to progenitors. Although expressed in parenchymal cells, possibly neurons, the high level of expression in the ventral VZ adds support to tanycytes as adult neural progenitor cells.

Hes5, an effector of the Notch signalling pathway, has been shown to be required for the maintenance of neural stem cell populations in both the embryo and the adult. Double mutants for *Hes1* and *Hes5* (to avoid any compensation) result in premature differentiation of embryonic radial glia into neurons, supporting a role for the Hes family in maintaining an undifferentiated radial glia population (Ohtsuka *et al.*, 2001; Basak and Taylor, 2007). The transgenic line Hes5::GFP has recently revealed expression in both quiescent and actively proliferating adult SGZ neural stem cells (section 1.1.3, page 34) (Lugert *et al.*, 2010). Considering the central role that Notch signalling plays in neurogenesis, and data that suggests that hes5-expressing cells are maintained as neural stem cells in the embryo and adult, Hes5::GFP presents a useful genetic tool to interrogate the 3rd ventricle of the adult hypothalamus. Importantly, GFP shows an extremely specific expression pattern in the adult, restricted to the alpha2-tanycyte subpopulation (figure 3.4). This is the first result that supports the presence of a neural stem cell population within the alpha-tanycytes alone. When Hes5::GFP expression is characterised along the adult anteroposterior axis, restricted expression is found throughout in addition to a difference in regional localisation. This data supports a population of neural progenitors that is distributed adjacent to the tuberal 3$^{rd}$ ventricle at the level of the median eminence. Furthermore, this may suggest alpha2-tanycytes show differences in location relative to the 3$^{rd}$ ventricle anteroposteriorly, or that Hes5::GFP is expressed in a heterogenous population of ependymal cells. Further investigation is required to definitively characterise the tanycyte subpopulations along the anteroposterior axis. This is of significance as recent studies apply the tanycyte terminology that was defined in the most medial region of the tuberal hypothalamus to other regions, which may lead to discrepancies between laboratories and misinterpretation.

Together with Six3 and Sox3 expression patterns, these data support an adult neural stem cell population within the alpha2-tanycytes and a population of uncommitted progenitors in the surrounding VZ, including alpha1-tanycytes and beta-tanycytes. In light of these results, the traditional tanycyte nomenclature based on position and barrier properties may not be appropriate in characterising a potential hypothalamic niche: future studies should aim to address the heterogeneity within tanycyte subpopulations as well as the degree of homogeneity between them in order to avoid dogmatic/semantic pitfalls.

### 3.3.2 Alpha-tanycytes display hallmark features of embryonic radial glia

Embryonic radial glial cells show definitive morphological characteristics including an RC2-positive basal projection and a single, apical primary cilium (section 1.1.5). Such features are not just structural identifiers, but are crucial contributors to the function of these embryonic neural stem cells, for example by ensuring cell-signalling propagation in the Shh pathway (Han *et al.*, 2008). Furthermore, these characteristics are shared by adult neural stem cells in the SVZ and SGZ, supporting their inextricable link with neural precursor status.

Tanycytes within the hypothalamic ependyma are a unique adult cell type, being recognisable by their long basal projections that extend to hypothalamic nuclei and the median eminence. The RC2 antigen, encoded by *Nestin,* is frequently used for the detection of radial glia, showing a remarkable degree of specificity in comparison to other intermediate filament proteins, such as Vimentin (Park *et al.*, 2009). RC2-positive cells can be observed at E15 in the hypothalamic VZ, and expression is maintained in cells of the ventral ependyma in the adult hypothalamus, corresponding to the position of both alpha2-tanycytes and beta1-tanycytes (figure 3.5B). While the Nestin antigen itself does not show tanycyte subtype specificity, the restricted RC2 expression pattern supports heterogeneity with regards to neural progenitor status within the tanycyte sub-classes. The maintained expression also suggests that alpha2- and beta1-tanycytes may be remnants of the embryonic RC2-positive population, and that radial glia-like cell-derivatives are maintained in the postnatal and adult hypothalamus.

Primary cilia are an additional defining characteristic of neural stem cells embryonically, and in adult niches when compared to multi-ciliated ependymal cells. A well-studied role of the primary cilia is that which is found in Shh-signalling, known to be involved in maintaining an undifferentiated state and regulating proliferation in neural stem cells. In the absence of Shh, most of the components of the Shh signalling pathway are localised to the cilium, whilst the downstream activator, smoothened (Smo), is inhibited from entering. Upon Shh binding to its receptor, patched, this complex is translocated away from the primary cilium, derepressing Smo and allowing entry of Smo into the cilium, where it can activate the pathway via the Gli transcription factors. The translocation of proteins around the cilium requires regulated intraflagellar transport by a number of proteins, including the small GTPase, Arl13b. Significantly, mice lacking Arl13b have abnormal Shh-signalling, as this protein has been shown to regulate the entry of Smo into the cilium (Larkins *et al.*, 2011). In addition, Arl13b-regulated cilia have been recently implicated in the organisation of the radial projection during development of the forebrain (Higginbotham *et al.*, 2013). An antibody raised against Arl13b is used here to identify the presence of primary cilia in order to determine whether the hypothalamus shares such features with the classic neurogenic niches (figure 3.6). Arl13b-positive signal can be seen in both the E15 and P35 ependymal cells, showing primary cilia morphology. In the adult, alpha-tanycyte regions and beta1-tanycyte regions display high numbers of Arl13b-positive primary cilia compared to beta2-tanycyte regions, supporting the RC2 analysis and independently identifying features of radial glia in cells within the alpha-tanycyte and beta1-tanycyte subtype. Significantly, the presence of Arl13b-positive primary cilia on tanycytes distinguishes them from terminally differentiated, multi-ciliated ependymal cells. Together these data support the presence of an adult neural stem/progenitor population within the alpha- and beta1-tanycyte subtype that maintains embryonic radial glia morphology.

### 3.3.3 Alpha-tanycytes are specified embryonically and are BrdU-retentive

BrdU labelling is a classic methodology to mark cells that have undergone proliferation, cells incorporating BrdU into their DNA during S phase of the cell cycle (Cavanagh *et al.*, 2011). A BrdU injection regime, performed over 24 hours for embryos at consecutive stages, confirmed a peak of proliferation at E15 and E16 followed by a significant decrease in the number of proliferating cells at E17 (figure 3.7A-C). Further, anti-PH3, used to quantify the number of mitotic cells at the point of fixation, supported the BrdU-labelling data and showed a further decrease in proliferation at E18 (figure 3.7D).

From the data above, I postulated that a subpopulation of adult tanycytes are embryonic radial glia cells that are maintained in an undifferentiated state in the VZ. In order to address this, a pregnant dam was injected with BrdU at E15 and E16 to allow incorporation of BrdU into VZ cells during their last round of division before proliferation significantly reduces. Retention of the BrdU-label in the VZ would suggest that either the cells had terminally differentiated and therefore not diluted the BrdU signal, or alternatively, that the cells remained undifferentiated and divided infrequently, characteristics of quiescent stem cells.

Conversely, no retention at the VZ would suggest cellular death, migration or multiple rounds of division that results in a dilution of BrdU within the DNA. Analysis of siblings at P0, P14, P35 and P49 revealed retention at the VZ at all ages. Importantly alpha-tanycyte regions showed high levels of BrdU at adult ages P35 and P49 (figure 3.8B,C). While the limitations of single thymidine labelling cannot disseminate a terminally differentiated cell from an undifferentiated progenitor in this paradigm, coupled with expression patterns of neural progenitor markers, these results support alpha-tanycytes as harbouring an infrequently dividing stem cell population. Future studies will utilise double thymidine incorporation, whereby a second thymidine analogue is incorporated during a later round of division to provide evidence that the cell is able to enter, exit and re-enter the cell cycle (Stoll *et al.*, 2011). Furthermore using transgenic animals that express a reporter in tanycytes will avoid co-staining problems associated with BrdU antigen retrieval, and will provide definitive evidence of BrdU retention in tanycytes specifically.

The observable regional differences in BrdU-retention between alpha-tanycyte regions and beta-tanycytes regions might suggest that beta-tanycytes originate largely from another cell-type that is not proliferative during the E15/E16 injection regime (figure 3.8B). Alternatively, if they were labelled during the regime and originate from the same embryonic location, it would suggest that beta-tanycytes undergo frequent postnatal proliferation. Indeed, this is supported by lineage-tracing studies that postulate beta-tanycytes as neurogenic cells in the juvenile mouse (Lee *et al.*, 2012a). The cellular relationship between alpha- and beta-tanycytes may therefore be one of direct lineage, with alpha-tanycytes being relatively quiescent stem cells and beta-tanycytes being more committed and proliferative progenitors; or could indicate two separate progenitor populations with different cell-cycle characteristics. In order to provide definitive evidence of an ancestral relationship between alpha- and beta-tanycytes, lineage-tracing studies, using conditional recombination to drive radial glia-specific reporter expression, could be combined with these data to determine the location and identity of any tanycytic progeny of embryonic VZ cells.

An additional result of interest is the difference in BrdU-retention depending on life-stage and gender. Both males and females show an increase in BrdU-positive cells in the postnatal VZ, at P14 (177.7 ±11.2 and 226 ±6.0 respectively), however after puberty (P35) the number of BrdU-positive cells in males decreases back to a level comparable to the P0 pups (70 ±4.6), whilst it is two-fold higher in females (144 ±8.3) (figure 3.8D). This is suggestive of a difference in proliferation associated with sexual maturation, for example by providing a degree of homeostatic flexibility that could be required for preparing the body for ovulation. Hypothalamic proliferation, related to maturation, is supported by evidence that finds a turnover of embryonic-born, appetite-regulating neurons, POMC and NPY, in the postnatal arcuate nuclei (McNay *et al.*, 2012). As females show twice the number of BrdU-positive VZ cells at p35, compared to males, this implies a sexual dimorphism in the preservation of postnatally-generated VZ cells. Considering the importance of tanycytes in regulating GnRH release for sexual maturation and maintenance of the estrous cycle (section 1.1.6; page 56), this could implicate a requirement for gender differences in tanycyte-continuance. Further investigations into the role of tanycytes in the male hypothalamus may elucidate the fate of postnatal VZ proliferation. Despite the comparison of siblings in this study, caution should be taken when interpreting these data, as the numbers of mice used does not provide an accurate statistical analysis. However, the results do support a sexually divergent, physiological role for hypothalamic VZ proliferation.

BrdU is frequently used for birth-dating studies, as the incorporation into mitotic cells provides an insight into the proliferative activity of tissues. However, it is important to note that no double-labelling with neuronal markers has been performed in these studies, therefore neurogenesis should not be implied. Furthermore, significant limitations exist with the use of BrdU injection regimes. BrdU is a mutagen, causing toxicity and cell death, and as it is a reporter for S phase of the cell cycle, it does not distinguish proliferation from other cellular processes that include DNA synthesis, for example apoptosis or DNA repair (Taupin, 2007). In these results, incorporation at the VZ is assumed to precede mitosis, however, as cells at other stages of mitosis will not be labelled, BrdU provides a relative number of proliferative cells and not an absolute number.

Proliferation was confirmed with phospho-histone3 (Hans and Dimitrov., 2001). Incorporation of BrdU can be mitogenic and lengthens the cell-cycle time; therefore caution should be taken when interpreting the proliferative rate of cells with BrdU inclusion into the DNA (Taupin, 2007). Of particular relevance to the experimental paradigm used here, BrdU can have teratogenic effects leading to developmental abnormalities and can cause cell death of newborn neurons, particularly in the embryonic CNS due to the lack of a blood-brain barrier (Taupin, 2007; Kolb *et al.*, 1999). Additionally, BrdU has been shown to reduce proliferation and cause senescence in neural stem and progenitor cells in vitro (Ross *et al.*, 2008), hence whilst care has been taken in interpreting these BrdU results, the extent to which BrdU incorporation has altered the proliferation and development of the hypothalamus is unknown. However, all embryos survived until the time of sacrifice, P49 latest, with no observable differences in phenotype compared to wild-type animals (data not shown). Despite the limitations, halogenated thymidine analogues remain widely used and a classical methodology for investigating proliferation in the context of neurogenic niches.

In conclusion, these data support an adult neural progenitor population within the tanycyte subtypes. The established markers Sox3, Six3 and Hes5 suggest subtle differences with respect to the extent of stem cell status between alpha- and beta-tanycytes. Cells within both subtypes display morphological characteristics of radial glia, including an RC2-positive basal projection and Arl13b-positive primary cilia. Finally, BrdU birth-dating and retention studies support alpha-tanycytes as an embryonically specified cell-type that become infrequently-dividing after E16; conversely, beta-tanycytes may include a proliferative progenitor population, potentially descended from the more quiescent alpha-subtype.

# Chapter 4

## Long-term lineage-tracing reveals alpha-tanycytes are self-renewing and multipotent in the adult hypothalamus

## 4.1: Introduction

With the advent of genetic engineering to indelibly mark cell types and follow their fate, it is insufficient to study neural progenitor populations retrospectively. Instead, lineage-tracing transgenic animals is a robust technique that allows for cell-specific and temporally-specific activation of a reporter. The use of a DNA cre-recombinase fused to a modified oestrogen receptor (CreER$^{T2}$), targeted to a stem cell-specific locus, presents an opportunity to selectively activate the reporter in adulthood (Novak, 2000). This is a significant advance over traditional systems that create gene products fused with a reporter that is not inducible, and that result in a convoluted interpretation of cell fate from adult niches.

Previous studies have used conditional recombination to mark and lineage-trace tanycytes in the hypothalamus; however, neither study used a subtype specific marker (Lee *et al.*, 2012a; Haan *et al.*, 2013). Both alpha- and beta-tanycytes express Fgf10 and Nestin, used by the authors, thus making it difficult to distinguish between the potential of these subtypes and preventing a definitive conclusion. Evidence supports alpha-tanycytes as infrequently dividing, neural progenitor cells with radial glial features. Therefore, I aimed to identify an alpha-tanycyte specific conditional driver in order to elucidate the fate and potential of this subtype in the adult. I present this work in this chapter. In addition, I provide BrdU infusion assays, performed by my collaborators, that aimed to explore potential regulators of the hypothalamic niche.

### 4.2.1: Recombination of Glast::CreER$^{T2}$ marks the alpha-tanycyte subpopulation

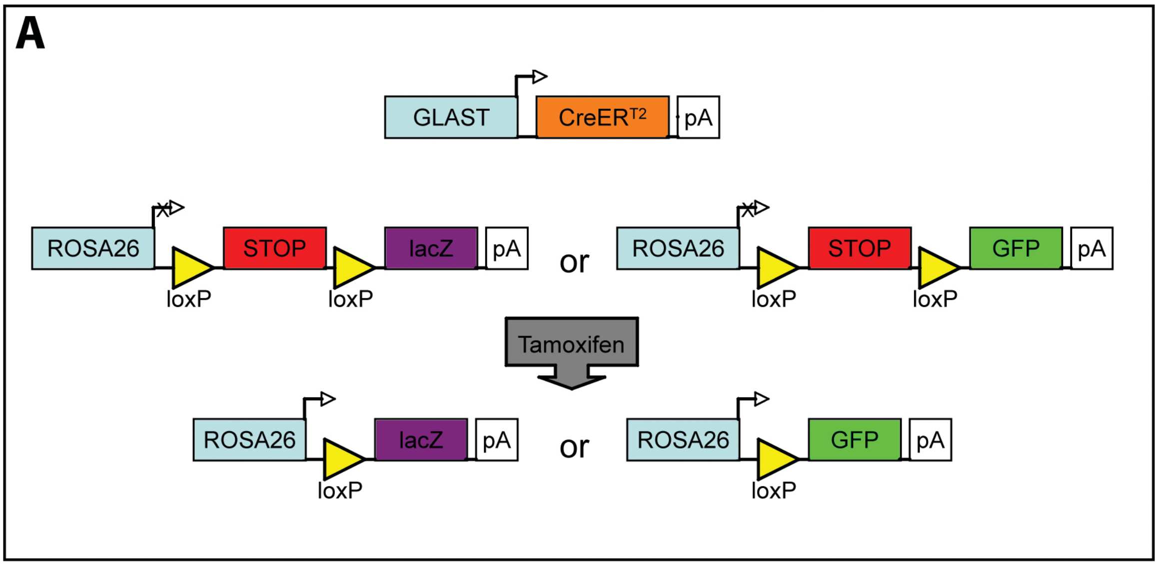
Glast, glutamate-aspartate transporter, is an established marker of neural stem cells in the SVZ and SGZ, and a conditionally-inducible Glast::CreER$^{T2}$ transgenic mouse has been generated to lineage-trace Glast-positive progenitors (Mori *et al.*, 2006). In this study, Glast::CreER$^{T2}$ mice have been crossed with mice that express a reporter (GFP or LacZ) ubiquitously under the

## Figure 4.1: Conditional recombination of Glast::CreER$^{T2}$ mice using tamoxifen

A. Schematic of tamoxifen-induced recombination of the Glast::CreER$^{T2}$ mouse transgenic line. Glast::CreER$^{T2}$ mice are crossed with Cre reporter mice expressing *LacZ* or *GFP* under the control of ubiquitous promoter, Rosa26. CreER$^{T2}$ is a cre-DNA recombinase fused with a modified oestrogen receptor, restricting the cre-recombinase to the cell cytoplasm. CreER$^{T2}$ is targeted to the Glast locus ensuring expression is specific to Glast-positive cells, including radial glia and astrocytes. In the presence of tamoxifen, cre-recombinase translocates to the nucleus where it mediates recombination by binding to two loxP sites that flank a neo cassette. Two recombinases bind per loxP site, and a tetramer forms that brings the two loxP sites together resulting in excision of the stop codon. Expression of the reporter gene is then turned on only in Glast::CreER$^{T2}$ -positive cells, allowing time-specific recombination conditional to radial glia and astrocytes. Due to the expression of the reporter under the ubiquitous promoter, when recombination has occurred, expression is maintained in any progeny, allowing lineage tracing of recombined cells.

B. Schematic illustrates the injection and chase regimes. 6-8 week old mice were injected with tamoxifen once-per-day for 5 days to induce recombination. Recombined mice expressing the GFP reporter were analysed at 5 days post-recombination and 9 months post-recombination (36 weeks). Recombined mice expressing the *LacZ* gene were analysed at 10 days post-recombination and 6 weeks post-recombination. Short-term analysis points are 5 and 10-day post recombination, 6 weeks is a mid-term analysis point, and 9 months is used as a long-term lineage tracing time-point.

C. Low power images of the tuberal hypothalamus of Glast::CreER$^{T2}$ mice that have undergone tamoxifen-induced recombination compared to those that received a vehicle injection. No GFP immunoreactivity is detected in non-induced animals, while activity can be detected in those that have undergone recombination. Scale bar represents 200$\mu$m.


A
GLAST
CreER$^{T2}$
pA
ROSA26
STOP
lacZ
pA
loxP
loxP
or
ROSA26
STOP
GFP
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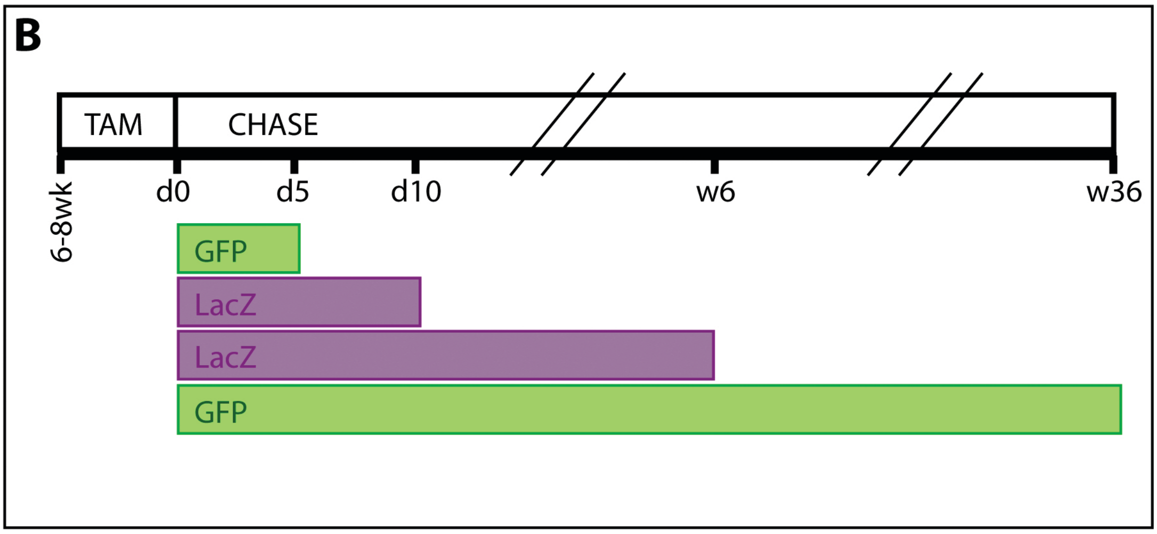
loxP
Tamoxifen
ROSA26
lacZ
pA
loxP
or
ROSA26
GFP
pA
loxP


B
TAM
CHASE
6-8wk
d0
d5
d10
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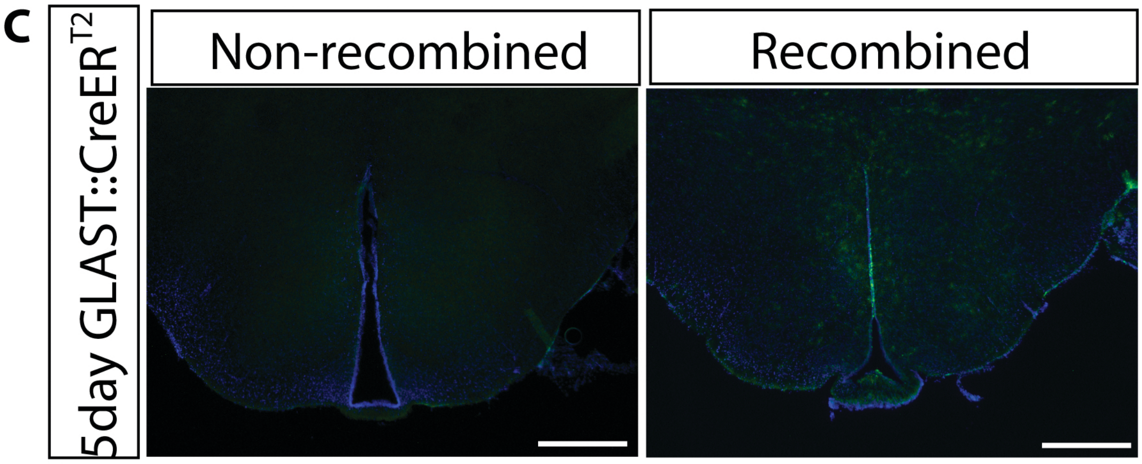
LacZ
LacZ
GFP


C
5day GLAST::CreER$^{T2}$
Non-recombined
Recombined

control of the ROSA26 promoter, including a floxed neomycin cassette which is excised in response to injection of oestrogen receptor antagonist, tamoxifen (figure 4.1A). This tool therefore ensures specific reporter expression in Glast-positive cells, and their progeny, that can be induced in adulthood. Previous studies have detected proliferation and neurogenesis in the beta-tanycytes of juvenile mice (Lee *et al.*, 2012a). However, other studies (Xu *et al.*, 2005; Perez-Martin *et al.*, 2010), and my own results showing embryonic progenitor characteristics within the alpha-tanycyte subpopulation (chapter 3), prompted me to investigate the potential of alpha-tanycytes in adults.

Mice were injected with tamoxifen at 6-8 weeks, an age considered to be adulthood. After tamoxifen-induced recombination, mice were chased to a short-term time point (5 and 10 days post recombination) in order to identify cells that were Glast::CreER$^{T2}$-positive (figure 4.1B). Comparing mice recombined with tamoxifen and vehicle alone (non-recombined) revealed no leaky expression of the GFP reporter in non-recombined mice. Conversely, GFP expression was detected in the tuberal hypothalamus of tamoxifen-induced Glast::CreER$^{T2}$ animals (figure 4.1C).

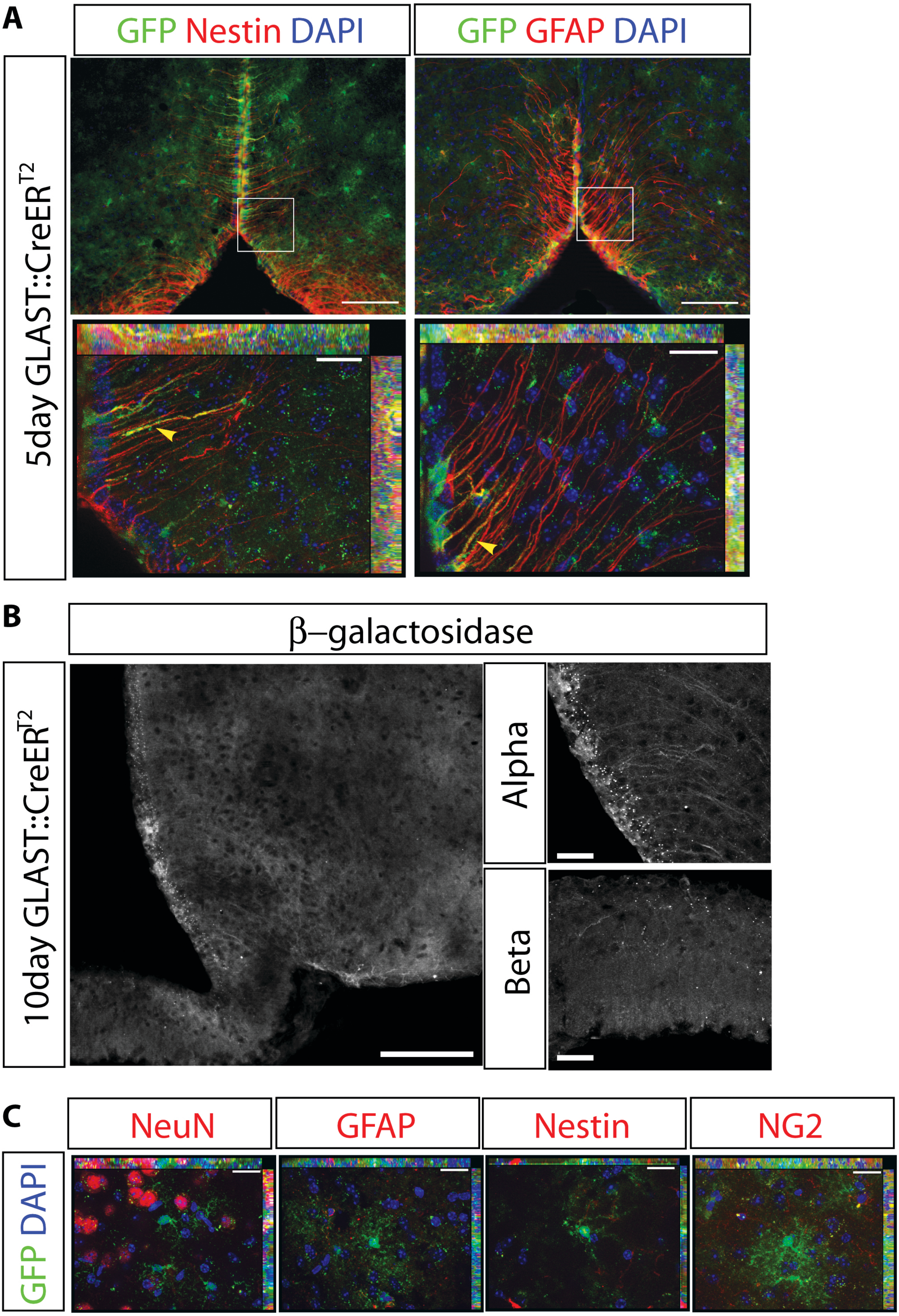
Labelling with the established tanycyte markers, Nestin and Gfap, revealed that reporter expression was detected within tanycytes (figure 4.2A). Anti-Gfap has been previously shown to distinguish between two alpha2-tanycyte subtypes, identifying a ventral and dorsal subgroup. Co-expression of Gfap and the GFP reporter supports Glast::CreER$^{T2}$ as marking dorsal alpha2 tanycytes. Since reporter expression is additionally detected immediately ventral to the Gfap-border (figure 4.2A), this indicates that reporter expression marks both dorsal and ventral alpha2-tanycyte subtypes. By contrast, recombined cells are not detected in the beta-tanycyte subtypes (figure 4.2B). Within the VZ of the third ventricle, reporter expression is thus restricted to the alpha-tanycyte subtype. These data confirm the Glast::CreER$^{T2}$ as an appropriate transgenic tool to study the potential of alpha-tanycytes, avoiding the caveats of previous studies that could not genetically distinguish between beta- and alpha-tanycytes.

## Figure 4.2: Glast::CreER$^{T2}$ specifically marks alpha-tanycytes in the ependyma of the third ventricle

A. Glast::CreER$^{T2}$ mice crossed with GFP reporter mice, analysed 5 days post-recombination. Transverse section: reporter activity is detected with anti-GFP; co-labelling with antibodies against the tanycyte markers Nestin and Gfap. DAPI is used to label cell nuclei. GFP is expressed within the ependyma and parenchyma; boxed region is presented as maximum intensity projection (MIP) below showing co-labelling of GFP with tanycyte markers (yellow arrows). Scale bar represents 100μm in top panel and 20μm in bottom panel.

B. Glast::CreER$^{T2}$ mice crossed with LacZ reporter mice, analysed 10 days post-recombination; reporter activity is detected with anti-β-galactosidase (Bgal). An image of the tuberal hypothalamus is shown alongside magnifications of alpha-tanycyte and beta-tanycyte rich region in the right-hand-side (RHS) panels. Expression of beta-gal is detected in alpha-tanycytes at 10 days post-recombination, while no expression is observed in the beta-tanycytes. Scale bar represents 100μm in left image and 20μm in RHS panel.

C. Glast::CreER$^{T2}$ mice crossed with GFP reporter, analysed 5 days post-recombination. Reporter activity is detected with anti-GFP and antibodies used to detect mature neurons (NeuN), astrocytes (Gfap) and progenitors (Nestin, Ng2) are used to identify parenchymal GFP-positive cells. No co-labelling is detected with NeuN, Nestin and Ng2 and while a small minority are Gfap-positive (Figure 4.5C), the majority do not - with established neuronal, astrocyte or progenitor markers. Scale bar represents 20μm.


A
GFP Nestin DAPI
GFP GFAP DAPI
5day GLAST::CreER$^{T2}$
B
β–galactosidase
10day GLAST::CreER$^{T2}$
Alpha
Beta
C
NeuN
GFAP
Nestin
NG2
GFP DAPI

Recent studies support a hypothalamic parenchymal progenitor population that is positive for chondroitin sulfate proteoglycan Ng2 (Robins *et al.*, 2013b), and that is currently considered to be an oligodendrocyte-precursor (Kang *et al.*, 2010). As reporter-positive cells are detected in the hypothalamic parenchyma in acutely-chased animals, it is important to characterise these cells, to prevent assumptions on the fate of progeny being attributed to the alpha-tanycyte subtype. Antibodies against established markers for neurons (NeuN), astrocytes (Gfap) and progenitor cells (nestin, Ng2) were used to identify the reporter-positive parenchymal cells (figure 4.2C). No co-expression was detected with NeuN, nestin or Ng2 suggesting they are neither progenitors nor neurons. Co-expression with Gfap was detected in a small population of reporter-positive parenchymal cells (figure 4.5C); however the majority of reporter-positive cells did not co-express Gfap. The identity of reporter-positive cells in the parenchyma therefore remains elusive, yet evidence suggests they do not have progenitor characteristics and are not neuronal, therefore for the purposes of this study the fate of reporter-positive cells after lineage-tracing can be attributed to the recombined tanycyte population.

### 4.2.2: Recombined Alpha-tanycytes generate tanycyte subtypes

To determine the fate of Glast::CreER$^{T2}$ -positive cells, recombined mice were chased for 6 weeks and 9 months (figure 4.1B) as previous studies suggest proliferation within the hypothalamic VZ is substantially less-frequent than in the classic neural stem cell niches, the SVZ and SGZ. The intent was to compare reporter-positive cells in short-term (5-10 days), mid-term (6 week) and long-term (9 months) chased animals, in order to observe whether the Glast::CreER$^{T2}$-positive population expands over time, i.e. generates progeny.

A striking expansion was observed in the extent of reporter-positive cells within the VZ between 5 days and 9 months post-recombination (figure 4.3A). Along the dorso-ventral axis, the number of VZ reporter-positive cells significantly increased from a mean number of 42.2 (±1.7), per 30μm hemi-section, to 73.7

(±4.7) (figure 4.3B). 35 field-of-views per hemi-section of the tuberal hypothalamus were analysed in detail for reporter-expression, and the results are summarised as heat-maps (figure 3.3C). These analyses reveal a spreading of reporter-positive cells along the dorso-ventral axis of the third ventricle and into the hypothalamic parenchyma. The expansion in reporter-positive cells gradually increases between the short-term (5-10 day), mid-term (6 week) and long-term (9 month) analyses points.

Comparison of reporter-positive cells according to tanycyte subtype, between 5 days and 9 months, reveals reporter-positive tanycytes in all subdomains after the long-term chase (figure 4.3D). This is in stark contrast to the restriction of recombination events primarily to the alpha2-tancyte subtype in acutely chased animals. An increase in the number of reporter-positive alpha2-tanycytes was observed from 25.8 (±1.6) cells, per 30μm hemi-section, to 35.6 (±2.9) cells after 9 months. The most statistically significant increases in VZ reporter-positive cells were detected in the beta-tanycyte (0.9±0.6 to 19.1±3.7) and alpha1-tanycyte (5.9±0.4 to 13.3±1.0) subregions (figure 4.3E). In order to ensure the change in reporter-positive cells was not due to a gross change in the number of VZ cells, DAPI-positive nuclei were quantified: this revealed no significant change in the total number of VZ cells (figure 4.3F).

Taken together, these results support Glast::CreER$^{T2}$-positive alpha-tanycytes as a population that generates cells fated to the beta, alpha2- and alpha1-tanycyte lineages. As no quantifiable difference was observed in the number of DAPI-positive nuclei along the dorso-ventral axis of the third ventricle VZ, the extent of reporter-positive cells at 9 months suggests that tanycytes are generated to replace older counterparts. Furthermore, these data confirm alpha-tanycytes as a progenitor population in the adult mouse hypothalamus.

### 4.2.3: Alpha-tanycytes are neurogenic and gliogenic

The observed expansion of reporter-positive cells away from the VZ into the hypothalamic parenchyma raises the possibility that alpha-tanycytes are able to

## Figure 4.3: The reporter-positive alpha-tanycyte populations expand and contribute to beta-tanycytes

A. Glast::CreER$^{T2}$ mice crossed with GFP reporter, analysed at 5 days post-recombination and the long-term time point, 9 months. Anti-GFP is used to detect reporter activity in the tuberal hypothalamus. The extent of reporter activity in the ependyma is observed to increase along the dorso-ventral axis. Scale bar represents 100$\mu$m.

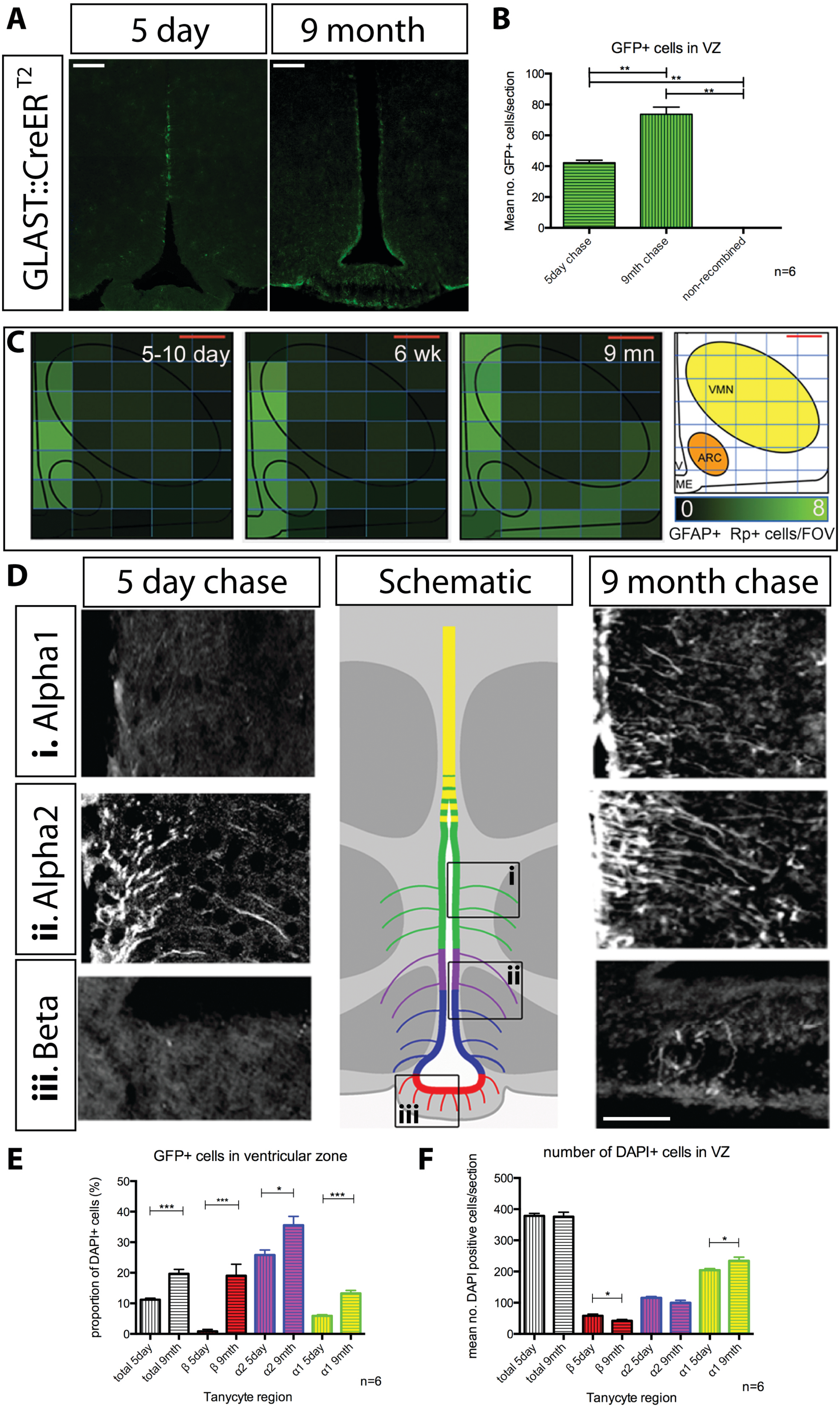
B. The mean number of GFP-positive cells in the VZ per 30$\mu$m hemi-section are quantified in mice 5 days post-recombination compared to 9 months post-recombination and non-recombined mice. No VZ GFP-positive cells are observed in non-recombined mice, while a statistically significant ($p<0.05$ =*) increase in GFP-positive cells is seen 9 months post-recombination compared to the VZ of acutely recombined mice ($n=6$).

C. Heat-map schematics are shown to illustrate the number of GFP-positive cells per field-of-view at 5 day, 6 weeks and 9 months post-recombination. After the short-term chase, GFP-positive cells are most abundant in the alpha-tanycyte region. The spatial distribution of GFP-positive cells gradually increases by the mid-term and long-term chase. Scale bar represents 100$\mu$m.

D. Glast::CreER$^{T2}$ mice crossed with the GFP reporter, analysed 5 days post-recombination or 9 months post-recombination. Reporter activity is detected with anti-GFP and the alpha1(i), alpha2(ii) and beta(iii)-tanycyte regions are compared. A schematic is used to indicate the tanycyte regions that are compared. All regions increase in the extent of tanycyte labeling between 5 days and 9 months, with tanycyte processes labeled. Scale bar represents 20$\mu$m.

E. The mean number of GFP-positive cells in the VZ of each tanycyte region are quantified in 30$\mu$m hemi-sections at 5 days post-recombination and 9 months post-recombination. A statistically significant increase in VZ GFP-positive cells is detected in the beta-, alpha2- and alpha1-tanycyte subregions ($p<0.05$ = *). The most significant increases are found in the beta- and alpha1-tanycyte subregions, while the alpha2-tanycyte subregion also shows an increase in the number of GFP-positive VZ cells (*n=6*).

F. The mean number of DAPI-positive cells in the VZ of each tanycyte region and in total are compared between 30μm hemi-sections of short-term and long-term chased animals. No significant difference is observed in total number of DAPI-positive ependymal cells between the sections analysed at the two time points, while a significant decrease can be found in the number of ependymal cells in the beta-tanycyte subregion, and a significant increase in the alpha1-tanycyte subregion ($p<0.05$ *=**). No change is observed in the alpha2-tanycyte subregion (*n=6*).

A
5 day
9 month
GLAST::CreER T2
B
GFP+ cells in VZ
Mean no. GFP+ cells/section
100
80
60
40
20
0
**
**
**
5day chase
9mth chase
non-recombined
n=6
C
5-10 day
6 wk
9 mn
VMN
ARC
V
ME
0
8
GFAP+ Rp+ cells/FOV
D
5 day chase
Schematic
9 month chase
i. Alpha1
ii. Alpha2
iii. Beta
i
ii
iii
E
GFP+ cells in ventricular zone
proportion of DAPI+ cells (%)
50
40
30
20
10
0
***
***
*
***
total 5day
total 9mth
β 5day
β 9mth
α2 5day
α2 9mth
α1 5day
α1 9mth
n=6
Tanycyte region
F
number of DAPI+ cells in VZ
mean no. DAPI positive cells/section
500
400
300
200
100
0
*
*
total 5day
total 9mth
β 5day
β 9mth
α2 5day
α2 9mth
α1 5day
α1 9mth
n=6
Tanycyte region

generate cell types other than tanycytes. To test this, I examined expression of doublecortin (dcx), to identify immature neurons, and neuronal nuclei (NeuN), to identify mature neurons, in order to determine the neurogenic potential of alpha-tanycytes. In addition, I examined expression of Gfap, in combination with astrocyte morphology, to assess the gliogenic potential of recombined cells in the Glast::CreER$^{T2}$ mice.

Co-expression of NeuN with the reporter was detected at 6 weeks and 9 months post-recombination (figure 4.4A), providing evidence that alpha-tanycytes have neurogenic potential. Indeed, at 9 months the reporter was expressed in NeuN-positive cells with distinct, bipolar, neuronal morphology.

Detailed analysis of the field-of-views, represented as heat-maps showing regions positive or negative for NeuN-positive/reporter-positive cells, revealed that at 6 weeks, double-positive cells were always in proximity to the initial, recombined alpha-tanycyte (figure 4.4B), and were in close association with the tanycyte processes. At the long-term (9-month) analysis point, double positive cells were additionally observed in both the ventromedial nuclei (4.4A) and arcuate nuclei of the tuberal hypothalamus. Conversely, no immature neurons with reporter expression were detected at 9 months, using anti-dcx, whereas dcx-positive reporter-postive (i.e. double-positive) cells were detected at 6 weeks (4.4C). Quantification of reporter-positive mature neurons reveals that the number of generated neurons is sparse, although statistically significant, after both mid-term and long-term analyses, the proportion of total GFP-positive cells that are double positive for NeuN being 1.8% (±0.1) and 1.4% (±0.4) respectively (figure 4.4D). Similarly, quantification of dcx-positive/reporter-positive cells reveals a significant increase in labeled immature neurons at 6 weeks compared to short-term analysis, accounting for 5% of reporter-positive cells (figure 4.4E). These results support alpha-tanycytes as a neurogenic population that, under these experimental conditions, generate a low number of neurons. Immature neurons are detected at 6 weeks, but are not detected at 9 months, suggesting these cells mature, migrate or undergo apoptosis. Indeed, at 6 weeks, the regions containing mature reporter-positive neurons overlap with the regions containing immature neurons, suggesting a transition to a

## Figure 4.4: Alpha-tanycytes have neurogenic potential in-vivo

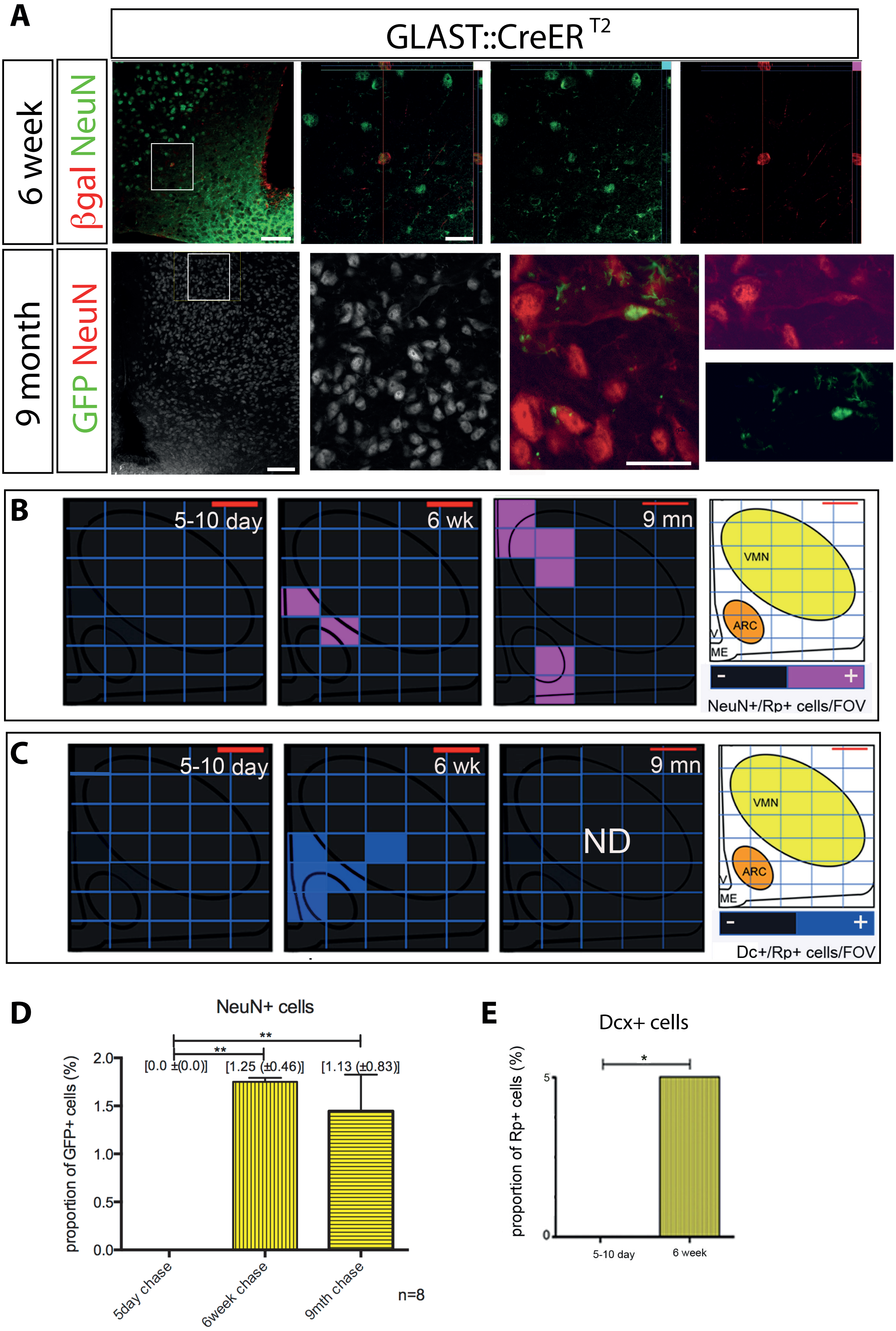
A. Anti-NeuN is used to detect mature neurons in mid-term and long-term chase time-points of Glast::CreER$^{T2}$ recombined mice, using anti-beta-gal and anti-GFP respectively to detect reporter expression. Confocal images are shown for 6 weeks post-recombination, and magnification of the boxed region shows co-labelling with neuronal marker. Apotome images are shown for 9 months post-recombination, and magnification of the boxed region shows region in which a cell with GFP expression co-expressed with NeuN, along with neuronal morphology, can be detected. Scale bar represents 100$\mu$m in the left hand side (LHS) images, and 20$\mu$m in RHS. 6 week post-recombination analysis was performed by Claudio Giachino and images are adapted from Robins *et al.* (2013).

B. Heat-map schematics illustrate the presence of reporter-positive/NeuN-positive cells within each field-of-view at 5 day, 6 weeks and 9 months post-recombination. At the short-term chase, double-positive cells are not detected. The spatial distribution of double-positive cells gradually spreads from a position adjacent to the alpha-tanycyte region at the mid-term, to a position within the ventromedial and arcuate hypothalamic nuclei at the long-term chase. Scale bar represents 100$\mu$m.

C. Anti-doublecortin (Dcx) was used to detect immature neurons that co-express the reporter. Heat-map schematics are shown to illustrate the presence of reporter-positive/Dcx-positive cells within each field-of-view at 5 day, 6 weeks and 9 months post-recombination. At the short-term chase and long-term chase, double-positive cells are not detected. At the mid-term chase, analysis indicates double-positive cells in positions adjacent to the alpha-tanycyte region and include the ventromedial and arcuate nuclei. Scale bar represents 100$\mu$m. 6 week post-recombination analysis with Dcx was performed by Claudio Giachino and images are reproduced from Robins *et al.* (2013).

D. The mean proportion of reporter-positive cells that co-label with NeuN are quantified at 5 day, 6 week and 9 month post-recombination. A statistically significant increase in the number of double-positive cells is seen at 6 week and 9 month compared to 5 day ($p<0.05$ =*), while no statistical difference is observed between 6 week and 9 month post-recombination. Actual mean cell counts and standard deviation are displayed in brackets (*n=8*).

E. The mean proportion of reporter-positive cells that co-label with Dcx are quantified at 5 day compared to 6 week post-recombination. A statistically significant increase in the number of double-positive cells is observed at 6 weeks ($p<0.05$ =*).


A
GLAST::CreER T2
6 week
βgal NeuN
9 month
GFP NeuN
B
5-10 day
6 wk
9 mn
VMN
ARC
V
ME
-
+
NeuN+/Rp+ cells/FOV
C
5-10 day
6 wk
9 mn
ND
VMN
ARC
V
ME
-
+
Dc+/Rp+ cells/FOV
D
NeuN+ cells
proportion of GFP+ cells (%)
2.0
1.5
1.0
0.5
0.0
**
**
[0.0 ±(0.0)]
[1.25 (±0.46)]
[1.13 (±0.83)]
5day chase
6week chase
9mth chase
n=8
E
Dcx+ cells
proportion of Rp+ cells (%)
5
0
*
5-10 day
6 week

mature cell type at the mid-term analysis in a location rich in tanycyte-processes. Despite the small number of reporter-positive mature neurons, their detection in hypothalamic nuclei at 9 months suggests a maintained function in the adult circuitry.

While these data provide evidence of neurogenic potential, the sparse numbers of reporter-positive neurons suggests that the reporter-positive parenchymal cells detected after a long-term chase are other cell types. To address this, anti-Gfap was used as a marker of astrocytes. Gfap-positive/reporter-positive cells, with stellate morphology, were detected at 9 months (figure 4.5A). Quantification revealed a 3-fold increase in the proportion of double-positive cells, from 15.7% (±2.0) at 5 days post-recombination to 44.8% (±2.7) at 9 months (figure 4.5B). However, as Gfap also marks dorsal alpha 2-tanycytes in the VZ, further quantification was performed to distinguish between VZ-tanycytes and non-VZ astrocytes (figure 4.5C). This analyses revealed that the number of Gfap-positive tanycytes doubled in 9 months, from a mean number of 10.3 (±0.6) double-positive cells per 30μm hemi-section, to 21.3 (±3.0) cells at 9 months post-recombination. At the same time, parenchymal Gfap-positive/reporter-positive astrocytes significantly increased in number, from 2.0 (±0.7) cells per 30μm hemi-section, to 18.5 (±1.6) cells between the short-term and long-term analysis. These results indicate that, under standard conditions, the majority of parenchymal cells generated from alpha tanycytes are astrocytes. However, although gliogenesis is more frequent than neurogenesis, the Glast::CreER$^{T2}$-positive tanycytes have the potential to generate both.

Together, the results from the lineage-tracing studies using the Glast::CreER$^{T2}$ transgenic mouse support the presence of a progenitor population within alpha-tanycytes, particularly the alpha2-tanycyte subtype. Inducing recombination with tamoxifen leads to restricted reporter expression in the alpha-tanycyte subpopulation, that expands dorsally and ventrally to generate tanycytes of alpha1-, alpha2- and beta1-subtypes, suggesting both self-renewal of recombined cells and generation of more committed tanycytes.

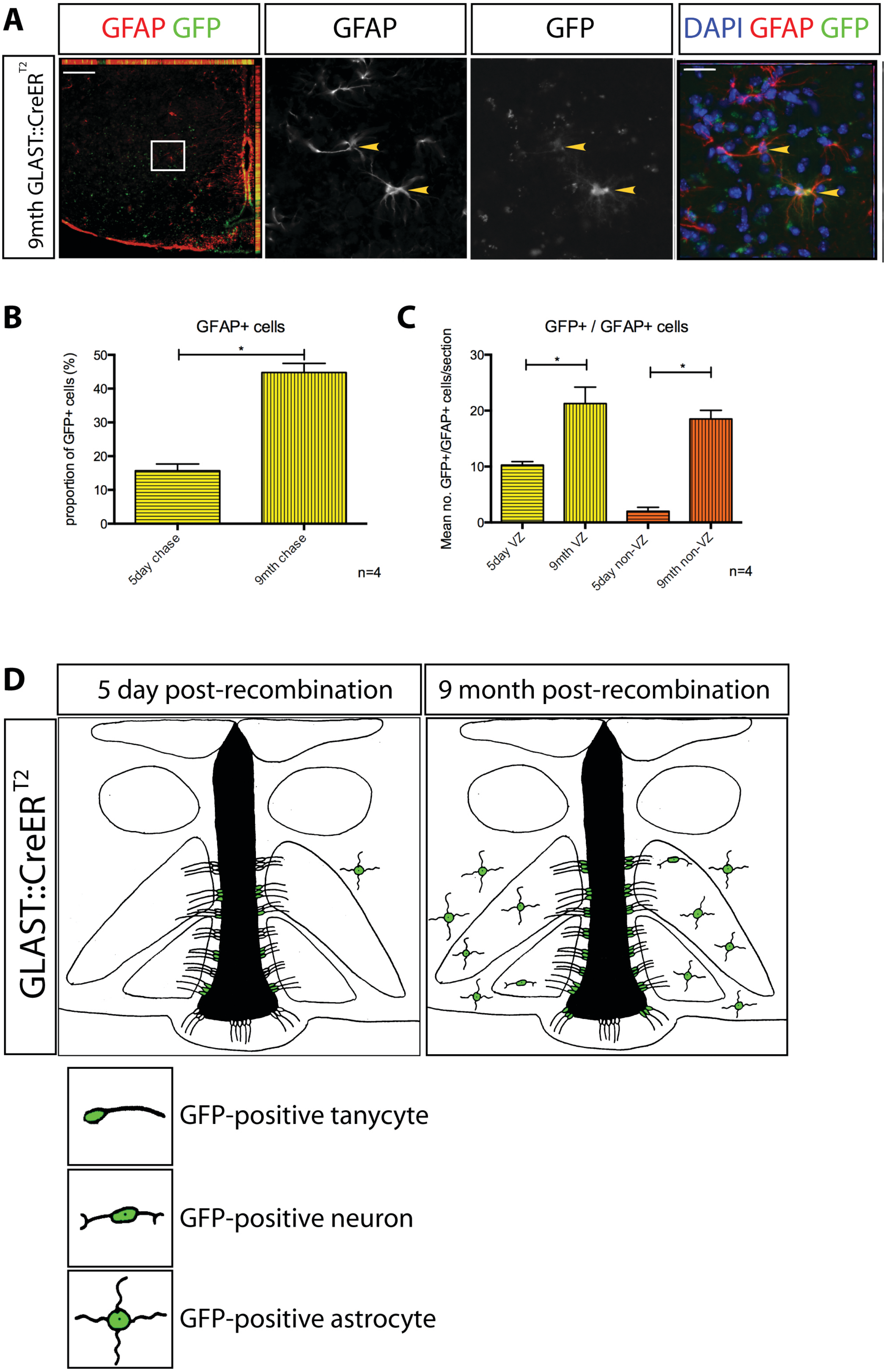
## Figure 4.5: Alpha-tanycytes generate astrocytes in mice housed under control conditions

A. MIPs are shown of Glast::CreER$^{T2}$ mice crossed with the GFP reporter and analysed 9 months post-recombination. Anti-GFP is used to detect reporter activity, and anti-Gfap is used to identify astrocytes. DAPI is used as a nuclear marker. Boxed region is magnified in RHS images. GFP-positive cells that co-express Gfap along with astrocyte morphology are shown (yellow arrowheads) alongside astrocytes that do not express the reporter (red arrowhead). Scale bar represents 100$\mu$m in LHS images and 20$\mu$m in RHS images.

B. The mean proportion of GFP-positive cells per 30$\mu$m hemi-section that co-express Gfap is quantified at 5 days and 9 months post-recombination. A significant increase is observed from 5 days to 9 months ($p<0.05$ =*) ($n=4$).

C. The mean number of GFP-positive cells per 30$\mu$m hemi-section that co-express Gfap is quantified at 5 days and 9 months post-recombination according to whether they are VZ or non-VZ cells. A significant increase in double-positive cells is observed from 5 days to 9 months ($p<0.05$ =*) in both the VZ and non-VZ ($n=4$).

D. Illustrations summarise lineage-tracing analysis. The extent of GFP expression in the tuberal hypothalamus of Glast::CreER$^{T2}$ mice at 5 days post-recombination and 9 months post-recombination is shown. In the short-term chase animals, GFP expression is concentrated in the alpha2-tanycyte region, with lower expression in the alpha1-tanycyte region and the parenchymal Gfap-positive astrocytes. Unidentifiable GFP-positive cells not shown. At 9 months post-recombination, GFP expression is detected in more alpha2-tanycytes. Additionally, alpha1- and beta2-tanycytes are now GFP-positive. GFP-positive astrocytes can be detected in increased numbers and sparse numbers of GFP-positive neurons are observed in the hypothalamic nuclei. Key shows cell types.


A
GFAP GFP
GFAP
GFP
DAPI GFAP GFP
9mth GLAST::CreER$^{T2}$
B
GFAP+ cells
proportion of GFP+ cells (%)
50
40
30
20
10
0
*
5day chase
9mth chase
n=4
C
GFP+ / GFAP+ cells
Mean no. GFP+/GFAP+ cells/section
30
20
10
0
*
*
5day VZ
9mth VZ
5day non-VZ
9mth non-VZ
n=4
D
5 day post-recombination
9 month post-recombination
GLAST::CreER$^{T2}$
GFP-positive tanycyte
GFP-positive neuron
GFP-positive astrocyte

Furthermore, the results provide evidence of neurogenesis from the alpha-tanycyte subpopulation, albeit at low levels in mice housed under control conditions for 6 weeks and 9 months. 5 days post-recombination, recombined astrocytes are observed at low numbers, indicating that Glast is also a marker of a mature astrocyte cell-type, as well as neural stem/progenitor cells in the hypothalamus. Significantly, the number of astrocytes generated from the genetically labelled niche over 9 months is far greater than the extent of neurogenesis, suggesting a preferential requirement for newborn glial cells in the unchallenged mouse hypothalamus (summarised in figure 4.5D).

### 4.2.4: Alpha-tanycytes proliferate in response to FGF2

The evidence above supports alpha-tanycytes as a multipotent neural progenitor population in the adult mouse hypothalamus, comparable to populations in the SGZ of the dentate gyrus and SVZ of the lateral ventricles. Basic Fibroblast Growth Factor (FGF2) has been shown to enhance neurogenesis in the SVZ, and is required for in-vitro culture of both SGZ and SVZ-derived neurospheres (Kuhn *et al.*, 1997; Reynolds and Weiss, 1992). The recognised mitogenic and neurogenic role of Fgf2 led us to investigate whether the different tanycyte subtypes respond differently to Fgf2, respective of their progenitor status. To address this, my collaborators infused adult wild-type mice with BrdU for 7 days, in the presence or absence of FGF2. Animals were then sacrificed immediately or after a 6-week chase. I include this data (figure 4.6), as it is pertinent to my own future studies (chapter 5,7).

Infusion of BrdU alone, followed by acute analysis, reveals sporadic incorporation throughout the parenchyma. In stark contrast, infusion with FGF2 leads to specific incorporation of BrdU within alpha-tanycytes (figure 4.6B). Quantification of BrdU incorporation in the median eminence (ME), VZ or SVZ indicates a significant increase in the number of BrdU-positive cells within the VZ, compared to the other regions (figure 4.6C). Additionally, antibody labelling for Gfap and vimentin to distinguish tanycyte subtypes confirms alpha2-tanycytes as responsive to FGF2, with the ventral alpha2-tanycyte subpopulation (Gfap-negative) showing the greatest incorporation of BrdU, and thus proliferation, followed by the dorsal alpha2-tanycyte subpopulation (figure 4.6D). Conversely, beta-tanycytes show no incorporation of BrdU in response to FGF2.

In order to address the long-term consequence of FGF2-induced proliferation, animals that had undergone the same infusion regime were chased for 6 weeks, and subsequently analysed. 6 weeks after BrdU infusion alone, very few BrdU-positive cells were observed in the hypothalamus. However, after BrdU infusion with FGF2, BrdU label was retained in a discrete location, particularly abundant in the Gfap-negative VZ (figure 4.6E). Quantification revealed a significantly higher number of BrdU-positive cells in the VZ and SVZ of mice that received FGF2 treatment (figure 4.6F). Quantification of BrdU-incorporation according to tanycyte subtype showed a significant increase in both alpha2-tanycyte subpopulations in response to FGF2 (figure 4.6G). These observations provide evidence that BrdU-positive cells in the SVZ are descended from alpha2-tanycytes.

Combining these BrdU studies with the lineage-tracing analysis presents strong evidence that the alpha2-tanycyte subtype is an FGF2-responsive, multipotent progenitor population in the adult mouse hypothalamus. Furthermore, my results that indicate alpha-tanycytes can contribute to the beta-tanycyte lineage combined with the specific proliferative response of alpha-tanycytes compared to beta-tancytes, suggests that a subpopulation of beta-tanycytes is derived from alpha-tanycytes. Together these data suggest that Glast::CreER$^{T2}$-positive alpha-tanycytes are uncommitted neural stem cells that generate cells of the neural lineage, and additionally, suggest a role for Fgf2 in regulating proliferation from the niche.

## Figure 4.6: The alpha2-tanycyte subpopulation is FGF2-responsive

A. Schematic of the BrdU injection and chase regime in weeks (w). 6-8 week old mice were infused with BrdU, along with FGF2 or vehicle alone, for 7 days. Animals were sacrificed acutely or after 6 weeks (*). David McNay performed these analyses and images are adapted from Robins *et al.* (2013).

B. Anti-BrdU was used to detect BrdU incorporation in acutely sacrificed mice that had received BrdU and vehicle alone (left hand panels) and mice that received BrdU and FGF2 (right hand panels). Anti-Gfap was used to identify tanycytes as shown in magnification of the boxed region. Sporadic BrdU incorporation is detected in the hypothalamic parenchyma and the SVZ in mice that had vehicle alone. In mice that received FGF2, strong BrdU signal is detected in the VZ of the alpha2-tanycyte subpopulation. Anti-vimentin staining reveals BrdU incorporation in the tanycyte cells (inset). Scale bar represents 100$\mu$m.

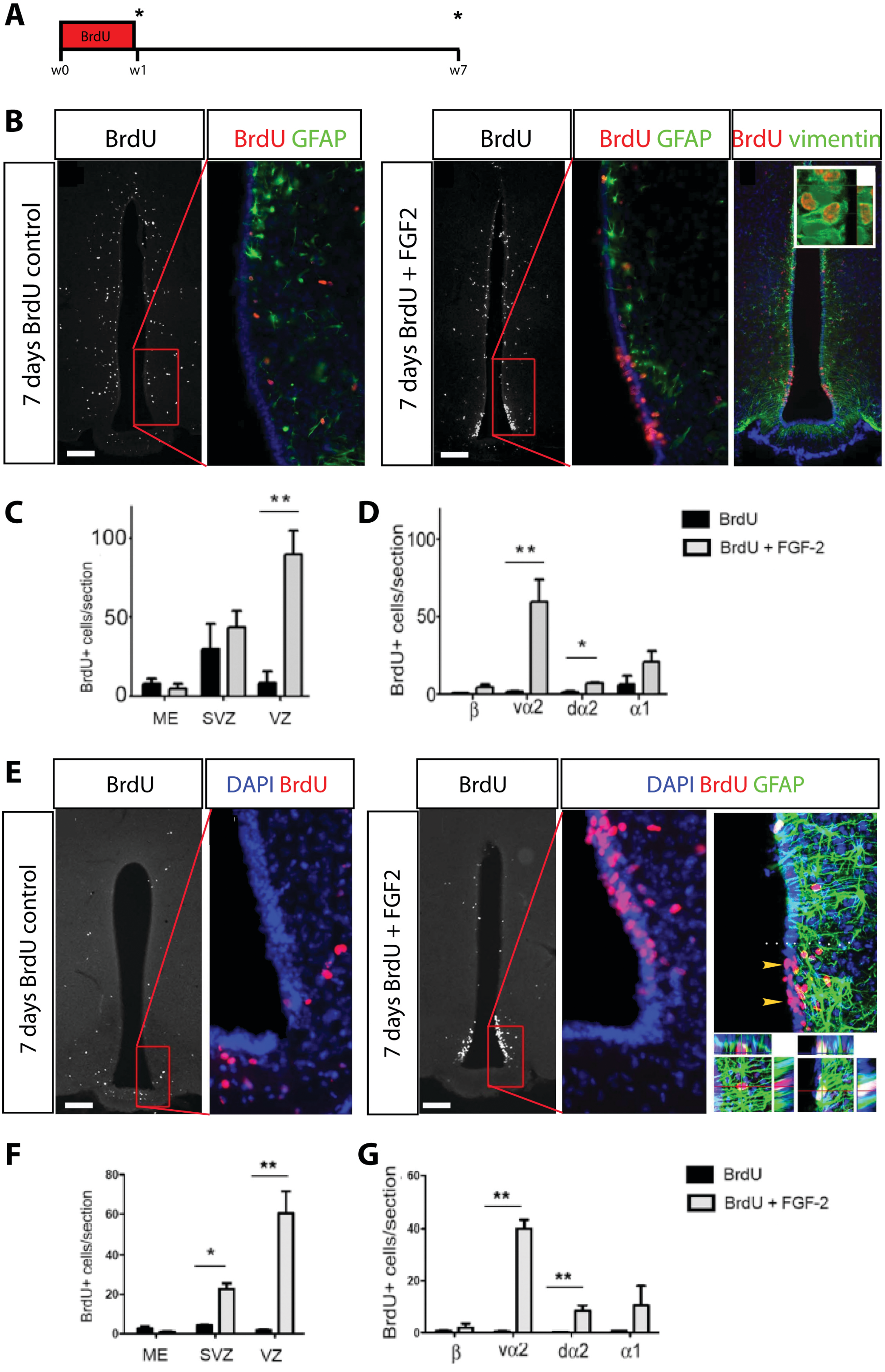
C. BrdU-positive cells are quantified according to location in median eminence (ME), ventricular zone (VZ) or subventricular zone (SVZ) in mice that received vehicle (black) or FGF2 (grey). The number of cells with BrdU incorporation is highest in SVZ with vehicle alone, while in response to FGF2 the VZ shows a significant increase in BrdU-positive cells ($p<0.05 = *$). No significant increase is quantified in the ME or SVZ in response to FGF2. (*n=3/6*).

D. BrdU-positive cells are quantified according to tanycyte subtype, as defined by co-labelling with anti-Gfap and anti-vimentin. Vimentin and location identifies alpha- and beta-tanycytes, Gfap distinguishes ventral (negative) and dorsal alpha2-tanycytes (positive). Mice that received vehicle are represented in black and mice that received FGF2 are represented in grey. In response to FGF2 an increase in BrdU-positive cells is observed in all tanycyte subtypes with a significant increase in both alpha2-tanycyte subtypes, and the most substantial statistical increase in the ventral alpha2-tanycytes ($p<0.05 = *$). (*n=3/6*).

E. Mice that received BrdU and vehicle alone (left hand panels) or FGF2 (right hand panels) are chased for 6 weeks and stained with anti-BrdU to detect label retention. DAPI is used as a nuclear marker. Little label retention is detected in mice that received vehicle alone. In response to FGF2, BrdU-positive cells are observed within the VZ and in the SVZ as shown in the magnification of the boxed region. Anti-Gfap reveals a large number of BrdU-positive cells in the ventral alpha2-subtype (yellow arrowheads), and BrdU-positive cells are detected in the SVZ (right hand inset) and dorsal alpha2-tanycytes (left hand inset). Scale bar represents 100μm.

F. BrdU-positive cells are quantified in chased animals according to location in median eminence (ME), ventricular zone (VZ) or subventricular zone (SVZ) in mice that received vehicle (black) or FGF2 (grey). Label-retention is low in mice that received vehicle alone. In response to FGF2, a statistically significant increase in the number of BrdU-positive cells within the SVZ and VZ is detected ($p<0.05$ =*), and no difference is detected in the ME. (*n=3/6*).

G. BrdU-positive cells are quantified in chased animals according to tanycyte subtype, as defined by co-labelling with anti-Gfap and anti-vimentin. In response to FGF2 a significant increase in BrdU-positive cells is observed in both alpha2-tanycyte subtypes ($p<0.05$ =*). An increase in the average number of BrdU-positive cells is observed in the alpha1- and beta-tanycyte subpopulation, although this is not statistically significant. (*n=3/6*).


A
BrdU
*
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BrdU vimentin
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C
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BrdU
DAPI BrdU GFAP
7 days BrdU + FGF2
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**
β
vα2
dα2
α1
BrdU
BrdU + FGF-2

## 4.3: Discussion

Lineage-tracing, using conditional recombination, is a state-of the-art technique to identify the stem-like potential of cell types. My previous data (chapter 3) supported a neural stem/progenitor population in the ventromedial ependyma of the 3$^{rd}$ ventricle, with a particular focus on the alpha-tanycytes. The aim of the research in this chapter was therefore to interrogate the alpha-tanycyte population with respect to their adult potential. In order to rigorously scrutinize the possible fate of alpha-tanycytes, and provide the first data that uses a conditional driver specific to a tanycyte subtype, I investigated the Glast::CreER$^{T2}$ transgenic line to determine its suitability (figure 4.1).

### 4.3.1: Glast::CreER$^{T2}$ specifically recombines in alpha-tanycytes in the third ventricle of the adult hypothalamus

As outlined in the Introduction (section 1.1.6), a number of earlier lineage-tracing studies suggested tanycytes harbour progenitor characteristics. In rat, non-genetic fate mapping studies suggested tanycytes may give rise to parenchymal neurons (Pencea *et al.*, 2001). More recently, in mouse, the use of conditional recombination at age-specific points, targeted to tanycyte-specific markers, has provided evidence that tanycytes are neurogenic when energy homeostasis is challenged (Lee *et al.*, 2012a). While these important studies support a physiological role for tanycyte neurogenesis, neither study defined an adult multipotent neural stem cell. Moreover, the genetic lineage-labelling studies performed to date have not examined the neurogenic potential of *subpopulations* of tanycytes. As outlined in the Introduction (section 1.1.6, page 59), Lee *et al.* (2012a) utilised a Nestin::CreER$^{T2}$ transgenic line and observed reporter positive neurons in the median eminence, supporting their conclusion that beta-tanycytes form a diet-responsive niche. However, Nestin is expressed by all tanycytes and thus the identity of the neurogenic tanycyte as the beta-tanycyte is an assumption based on location and a lack of migration, not necessarily the inherent benefit of using a transgenic animal. More recently, Haan *et al.* (2013) used an Fgf10::CreER$^{T2}$ line to examine the neurogenic potential of Fgf10-positive tanycytes; however, both their study and our previous data (section 1.1.6) provide evidence that both alpha- and beta-tanycytes express *Fgf10*, again impeding a direct conclusion to the potential of the different tanycyte cells. Furthermore, targeting the cre-recombinase to the Fgf10 locus could lead to inconsistencies if the gene expression of this Fgf changes depending on age, diet or other physiological variables.

In contrast, in this study I identified Glast::CreER$^{T2}$ to be specifically recombined in alpha-tanycytes within the ependyma of the 3$^{rd}$ ventricle, and in particular alpha2-tanycytes (figure 4.2). This has allowed me to distinguish alpha-tanycyte potential from beta-tanycyte potential, a characterisation that has not yet been accomplished.

In addition, I induced recombination between 6 and 8 weeks of age, a point that is considered to be categorically adult in the mouse. My chase regimes then included short, mid and long-term analysis points, with a maximum chase of 9 months. This is in stark contrast to Lee *et al.* (2013) who induced recombination at postnatal day 4, and Haan *et al.* (2013) who induced recombination at 4 weeks of age. The caveat to inducing recombination at these early time points is that it obscures our understanding of adult neural stem cell potential, by including neurogenesis that occurs postnatally and during puberty. It is recognised that the challenges upon perinatal and juvenile animals stimulate the production and integration of newborn neurons, and while studies that investigate these processes are of significant interest, they should not be considered adult (Wei *et al.*, 2011). Adult neural stem cells are cells that retain multipotency after adolescence and into old age, where progeny contributes to repair, refinement and replacement of the circuits that were established in the embryonic and juvenile organism. My study was therefore designed to interrogate alpha-tanycyte potential in the adult hypothalamus.

### 4.3.2: Glast::CreER$^{T2}$-positive alpha-tanycytes contribute to the tanycyte lineages

My data provide evidence that alpha2-tanycytes generate tanycytes of the alpha2-, alpha1- and beta1-tanycyte subtype (figure 4.3), based on the location of reporter-positive cells in the ependyma of 6 week- and 9 month-chased animals, and provide the first evidence that alpha2 tanycytes form a neural stem cell within a hypothalamic niche (hereafter referred to as the niche). The increase in alpha2-tanycyte reporter-positive cells over time suggests self-renewal of this cell type, an archetypal feature of adult neural stem cells. However, as additional antibody staining against cre-recombinase has not been performed, it is not possible to definitively conclude the net increase in alpha2-tanycyte reporter-positive cells is self-renewal. Instead, the recombined population may be generating a tanycyte cell type that has differentiated roles, for example in regulating neuroendocrine release. As the heterogeneity within alpha2-tanycytes has not yet been addressed, it is possible that neural progenitor status and physiological function may be roles harboured by discrete populations or by the same. For this reason, while self-renewal is detected, it remains possible that some alpha2-tanycyte progeny are progenitors, rather than stem cells.

Lineage-tracing the Glast::CreER$^{T2}$-positive population also reveals reporter-positive beta1-tanycytes after 6 weeks and 9 months. These data suggest a population of beta1-tanycytes are descended from alpha2-tanycytes, and may indicate that the known neurogenic response of beta-tanycytes in young animals could be mediated by alpha2-tanycytes. Indeed, the classical view of neurogenic niches identifies a neural stem cell population, and a committed progenitor population (Basak and Taylor, 2009). If this view were to be adapted to the hypothalamus, alpha2-tanycytes would provide a neural stem cell population that gives rise to committed progenitors, including the beta1-tanycytes. Reporter-positive alpha1-tanycytes were also observed to increase in number, suggesting a bidirectional production of cells along the dorso-ventral axis of the 3rd ventricle ependyma, centred around alpha2-tanycytes. Evidence from the SVZ of the lateral ventricles and the SGZ of the dentate gyrus indicates that resident neural stem cells are able to regenerate their niche after destruction of niche cells (Doetsch *et al.*, 1999a; Seri *et al.*, 2001). Similarly, 9 month chased animals have reporter-positive cells throughout the VZ, supporting recombined alpha2-tanycytes as being able to contribute to their niche. We show here that, in the unchallenged mouse, alpha2-tanycytes constitutively generate other tanycyte subtypes.

Intriguingly, in the intestinal crypts of the gut, stem cells at the base generate proliferative progenitors that progressively differentiate in a columnar stream towards the tip of the intestinal villi, giving rise to endocrine cells and absorbing/secreting enterocytes (Simons and Clevers, 2011), reminiscent of the function of hypothalamic ependymocytes and differentiated tanycytes. Although the hypothalamic niche and the intestinal crypt niches descend from different embryonic germ layers, and are undoubtedly diverse, the commonality of a basal niche and ascending lineage commitment and differentiation suggests a common strategy to replace cells at the interface of fluids and tissue.

Finally, the absence of reporter-positive beta2-tanycytes in 9 month-chased animals, but the presence of the reporter in beta1-tanycytes, suggests these two subtypes have different origins. Previous studies in the Placzek lab have identified a population of label-retaining neurospherogenic stem-like cells that exist in a collar around the developing infundibulum, termed ‘collar cells’, which give rise to progenitors in the embryonic chick hypothalamus and infundibulum (Pearson *et al.*, 2011; Placzek and Pearson, 2013). In the adult mouse ventromedial hypothalamus, alpha2-tanycytes are present in a location synonymous with the embryonic collar cells, providing tantalizing support to a maintained population of neural stem-like cells throughout life. Fate-mapping studies in the embryonic chick demonstrate that collar cells and the most medial hypothalamic tissue are derived from different embryonic origins, the most anterior floor plate and the adjacent floor plate respectively (Placzek and Pearson, 2013). The differential distribution of reporter-positive cells in beta1- and beta2-tanycytes may imply that beta1-tanycytes are collar cell descendants, while beta2-tanycytes may originate from the more posterior floor plate or the oral ectoderm of Rathke’s pouch, which combines with the developing hypothalamus to generate the anterior pituitary. Considering that the median eminence, the site of beta-tanycytes, is an anterior protrusion to the pituitary, future studies will address the developmental origins of these adult cell types.

### 4.3.3: Glast::CreER$^{T2}$-positive tanycytes are multipotent.

The detection of reporter-positive tanycytes after mid- and long-term chase, supports Glast::CreER$^{T2}$-positive alpha2-tanycytes as a stem/progenitor population that can generate specialized glial cells of the ependyma. As neural stem cell populations in the SVZ and SGZ are neurogenic, I analysed reporter-positive parenchymal cells for neuronal marker expression, NeuN (Mullen *et al.*, 1992), in order to determine the frequency of neurogenesis in the hypothalamic niche (figure 4.4). Reporter-positive neurons were not detected in acutely analysed animals, but were detected in mid- and long-term chased animals. At 6 weeks and 9 months post-recombination the proportion of reporter-positive neurons was 1.8% and 1.4% respectively (figure 4.4D). This provides evidence of the neurogenic potential of recombined tanycytes. However the low numbers indicate neurogenesis is infrequent in the unchallenged mouse.

The low level of neurogenesis is further supported by detection of the immature neuronal marker, doublecortin (Brown *et al.*, 2003), in 8% of reporter-positive cells at 6 weeks post recombination (figure 4.4E). The apparent decrease in immature neurons to mature neurons suggests that the hypothalamic environment is not conducive to neuronal differentiation under these conditions. Furthermore, while doublecortin is traditionally used to detect immature neurons, recent studies provide evidence that doublecortin is not specific to the neuronal lineage, as it can be detected in mature astrocytes of the human neocortex (Verwer *et al.*, 2007). Therefore the numbers of reporter-positive immature neurons could potentially be lower. However, the presence of mature reporter-positive neurons in the ventromedial and arcuate hypothalamic nuclei at 9 months is suggestive of a functional role for the few neurons that did differentiate and survive. Future studies should use patch-clamping techniques in brain slices or *c-fos* expression to confirm activity of reporter-positive neurons (Hoffman, 2002).

In comparison to neurogenesis, the frequency of parenchymal astrocyte production is far more common under these experimental conditions, an 8-fold increase in the mean number of reporter-positive astrocytes detected after 9 months compared to 5 days post-recombination (figure 4.5C). The number of reporter-positive neurons accounts for ~1.5% of reporter-positive cells at 9 months, while Gfap-positive/reporter-positive cells account for ~45%, leaving 43.5% of reporter-positive cells unaccounted for using these markers. These remaining cells are likely made up of Gfap-negative tanycytes.

While recombination of Glast::CreER$^{T2}$ mice leads to reporter activity in alpha-tanycytes, reporter-positive cells are also detected in the parenchyma of acutely-chased animals. These could not be identified as progenitors (Nestin, Ng2) or neurons (NeuN) (figure 4.2C), and only a small number were positive for the astrocytic marker, Gfap (figure 4.5C). This indicates that Glast marks a small population of parenchymal astrocytes as well as the prospective neural progenitor cells in the VZ, supporting observations from other studies that utilise this transgenic line (Mori *et al.*, 2006). Although I was unable to identify the remaining reporter-positive cells as parenchymal progenitors, it is possible that they may produce new cells in the adult. However, new reporter-positive cells were consistently detected in close proximity to the ventricular zone in 6 week chased animals (figure 4.3C), rather than within the parenchyma. Furthermore, there was no significant change in the total number of reporter-positive cells at 5 days compared to 9 months (data not shown), suggesting the number of unidentifiable reporter-positive cells decrease over time, while concomitantly, new cells are generated from the Glast::CreER$^{T2}$-positive tanycytes.

Together, these data provide strong evidence that Glast::CreER$^{T2}$-positive alpha-tanycytes are gliogenic in the unchallenged mouse, generating tanycytes and parenchymal astrocytes, as well as potentially self-renewing. Neurogenesis, conversely, is rare under these conditions. Considering the environmental influence on hippocampal neurogenesis (section 1.1.4), including the environmental enrichment-induced increase in newborn neurons (Kempermann *et al.*, 1997b), and the physiological role for the hypothalamus in controlling homeostasis, it is unsurprising that neurogenesis is low in this experimental paradigm. Studies that detect neurogenesis at higher levels in the hypothalamus have routinely used diet or fasting to manipulate energy homeostasis (Kokoeva *et al.*, 2005; McNay *et al.*, 2012; Lee *et al.*, 2012a; Haan *et al.*, 2013). Of particular interest, Haan and colleagues (2013) identify a number of reporter-positive neurons one month after recombination in Fgf10::CreER$^{T2}$ mice. A subset of these neurons express Neuropeptide-Y, which has orexigenic properties, and reporter-positive parenchymal cells are subsequently activated in response to fasting. As Fgf10 is expressed by alpha-tanycytes, as well as beta-tanycytes, these neurogenic findings support the lineage-tracing data of Glast::CreER$^{T2}$ mice, with the neurogenesis I observe potentially originating from a population that expresses both Glast and Fgf10. Alternatively, ventral Fgf10-positive tanycytes that do not express Glast may also represent an energy-responsive neurogenic population that are excluded in this study.

In contrast to these physiological investigations, we focus on the intrinsic potential of alpha-tanycytes in the absence of external manipulation. As such, the recombined mice in our study are provided a standard and consistent diet, as well as a controlled light/dark-cycle and temperature. Energy-balance, thermoregulation and circadian cycles are well established to be under the control of hypothalamic circuits; therefore in the absence of natural and wild variation in season, temperature and food source, the plasticity provided by newborn neurons to a circuit controlling homeostasis may not be required. The comparably higher-production of astrocytes could support neurons within the networks, whose inherent plasticity mediated by structural dynamics and gene expression is sufficient to compensate for minimal variation in the environment during the chase regime.

An additional reason for the low levels of neurogenesis in conditionally induced mice, receiving no physiological stimulus, could be an artefact created by tamoxifen injection. Oestrogen has an influence within the hypothalamus, levels of which are frequently dependent upon the reproductive axis. The action of oestrogen on hypothalamic neurons has significant consequences on the synthesis and secretion of many downstream hormones including prolactin

(Shupnik, 2002), shown to influence proliferation in the SVZ of the lateral ventricles (section 1.1.4, page 42) (Larsen and Grattan, 2010). Moreover, oestrogens themselves have been shown to influence proliferation and neurogenesis within the SGZ of the dentate gyrus (Tanapat *et al.*, 1999; Fester *et al.*, 2006). When the oestrogen receptor antagonist, tamoxifen, is injected to induce recombination of Glast::CreER$^{T2}$-positive cells, via nuclear translocation of the recombinase, the antagonistic action could have off-target effects. Endogenous oestrogen receptors may be inhibited, and thus a response that would naturally induce neurogenesis after receptor activation would be diminished. As the fate of progenitor cells is in a delicate balance, dependent on factors that influence the niche, the antagonism of oestrogen receptors may provoke progenitors to generate astrocytes at the expense of neurons. Future studies will identify the influence of steroids such as oestrogen upon the hypothalamic niche. However, while tamoxifen injection may account for an initial change in neurogenic rate, it is unlikely to have a continued effect months after recombination.

In conclusion, these lineage-tracing studies identify a population of alpha2-tanycytes as adult neural stem/progenitor cells in the hypothalamus, constitutively generating tanycyte cell-types and astrocytes. These data support beta1-, alpha1- and alpha2-tanycytes as having the same origins, with the alpha2-tanycyte being the developmentally older progenitor maintained into adulthood. Furthermore, my studies show that alpha2-tanycytes are neurogenic, albeit producing few neurons in the unchallenged mouse. The specific recombination therefore demonstrates alpha-tanycytes as a critical component of the hypothalamic niche.

### 4.4.4: Alpha2-tanycytes are Fgf responsive

My lineage-tracing analysis provides strong evidence that a population of alpha2-tanycytes are neural stem/progenitors in the adult hypothalamus. Previous studies from the Placzek lab have identified endogenous Fgf expression patterns, localised to the alpha2-tanycyte subtype, namely *Fgf10* and *Fgf18* (section 1.1.6, figure 1.9). Both these ligands act through shared Fgf receptors with Fgf2, a growth factor that is required for in-vitro maintenance of neural stem cells and that enhances neurogenesis in the SVZ of the lateral ventricles (section 1.1.2, page 18). Considering the specific expression pattern of endogenous Fgfs, and the role of Fgf2 in a classically defined niche, FGF2 was infused into adult animals in order to identify whether hypothalamic niche cells were responsive to this factor in-vivo, in support of previous studies (Xu *et al.*, 2005) (figure 4.6: provided by D.McNay and M.Kokoeva).

Infusion of FGF2 with BrdU results in a striking increase in BrdU incorporation, compared to BrdU alone, that is largely specific to the alpha2-tanycyte subtype, and is especially high in the ventral alpha2-subtype (as defined by a lack of Gfap expression). After a 6 week chase, BrdU is retained within the alpha2-tanycytes; in addition, there is an increase in BrdU-positive cells within the adjacent SVZ, compared to controls. These results indicate that alpha2-tanycytes, and in particular, ventral alpha2-tanycytes, proliferate in response to FGF2 infusion and that incorporated-BrdU is not diluted over the chase period. Retention of BrdU therefore suggests proliferative cells remain in the VZ. The result of FGF2-induced proliferation appears to include a progression of progeny from the VZ to the SVZ and into the parenchyma along tanycyte processes (figure 4.6E right hand panels). It is, of course, possible that this apparent cell migration is not an observation of a dynamic course, but instead, that cells in the SVZ and parenchyma incorporate BrdU in response to FGF2, independently of an alpha2-tanycyte origin. However, as no significant difference was seen in the number of SVZ BrdU-positive cells in response to FGF2 immediately after infusion, these data support an FGF2-induced proliferative response in alpha2-tanycytes, the progeny of which migrate through the SVZ and into the hypothalamic parenchyma.

This investigation elucidates Fgf signalling as a regulator of proliferation within the hypothalamic niche, similar to the SVZ of the lateral ventricles. Coupled with the restricted expression patterns of a number of Fgfs to the alpha2-tanycyte subtype (and beta-tanycytes in the example of *Fgf10*), these data support endogenous Fgfs as influencing cell-cycle dynamics within the tanycyte niche.

The low levels of incorporation of BrdU in the absence of FGF2 infusion could suggest endogenous factors do not influence proliferation; however, transcription and translation of the gene into the protein product could change depending on physiological pressures that are controlled in these experimental conditions. Further studies will elucidate the response of niche cells to endogenous Fgf ligands (see chapters 5 and 7).

Taking the lineage-tracing data with the FGF2 infusion experiment, it is possible to conclude that alpha2-tanycytes are an Fgf-responsive neural progenitor cell in the adult hypothalamus. This provides the first demonstration of the specific potential of alpha-tanycytes and demonstrates a potential role for localised and endogenous factors. The finding that FGF2 induces proliferation in a restricted population of tanycytes, the alpha2-subtype, further substantiates the lineage-tracing evidence of alpha2-tanycytes as a multipotent progenitor.

# Chapter 5

## Characterisation of the hypothalamic niche using the in-vitro neurosphere assay

## 5.1: Introduction

Since 1992, when Reynolds and Weiss showed that isolated SVZ cells from the adult mouse striatum could generate neurospheres in culture, the neurosphere assay has been routinely used to ascribe neural stem cell characteristics to cells from the central nervous system. The formation of neurospheres in-vitro is accepted to provide an indication of the proliferative potential of neural stem/progenitor cells in-vivo. Indeed, studies have identified that adult SVZ neurospheres can be transplanted into the embryonic nervous system, where they contribute to development and maintain the multipotency that they display in the neurogenic zones of the adult (Neumeister *et al.*, 2009). Furthermore, serial transplantation of neural stem cells, via an in-vitro neurosphere expansion phase, reveals that they retain self-renewal potential**.** Hence, the neurosphere assay provides a valuable tool to assay neural stem cell identity in culture.

Although serial transplantation (as first used to define haematopoietic stem cells) provides the gold standard evidence of neural stem cell features, it is generally considered that neural stem cell potential can be characterized through a less difficult in-vitro assay: determining a cell's long-term passaging ability, as well as its potential to differentiate into multiple neural lineages after growth-factor removal. These features suggest the presence of a self-renewing, multipotent stem cell. By contrast, a transit-amplifying progenitor would form only primary or secondary neurospheres (Conti and Cattaneo, 2010). In this chapter I present experiments using the neurosphere assay that support my in-vivo characterisation of the hypothalamic niche. In addition, I assess the merit of the neurosphere assay in investigating a potential physiological response of the hypothalamic niche.

## 5.2.1: Assessment of optimal plating density for neurosphere growth

The neurosphere assay investigates the nature of neural stem/progenitor cells in-vitro, in the absence of the in-vivo niche conditions. Tissue is dissociated enzymatically and physically, and then plated as single cells at clonal density to assess neurosphere formation from a single cell. A balance is required: a low plating density will generate insufficient neurospheres to utilise in experiments and for subsequent passaging; a high plating density will lead to aggregation and non-clonal spheres. Neurosphere formation from the classically-defined neurogenic regions, the SVZ of the lateral ventricles and SGZ of the dentate gyrus, requires plating at a clonal density of 10 cells per microliter (10 cells/μl) (Giachino *et al.*, 2009). However, as I have shown in chapters 3 and 4, proliferation and neurogenesis in the hypothalamus is considerably less frequent than in the SVZ and SGZ, therefore the cell-cycle dynamics may also be different in-vitro. I therefore set out to establish the optimal plating density for hypothalamic neurosphere growth.

Dissociated tertiary neurospheres, considered a less heterogenous population compared to primary and secondary neurospheres, were plated at four different densities, including the established clonal density for the SVZ and the SGZ: 7, 10, 13 and 16 cells/μl (figure 5.1). Neurospheres that were generated from the different densities were dissociated and the concentration of cells was then quantified and calculated using a haemocytometer. The total cell number at the end of culture was divided by the total cell number at the beginning of culture, and the fold increase in cells is plotted in order to evaluate whether density affects proliferation (figure 5.1A). A downward trend in fold-increase was observed with increasing density of cells plated; furthermore, cells plated at higher densities were found to have a larger standard deviation from the mean. Plating cells at densities of 13 and 16 cells/μl is therefore an inefficient density for proliferation and results in large inconsistencies in cell number. An indication of the amount of neurospheres available for subsequent processing or passaging is also provided (figure 5.1B). This shows that plating cells at densities of 13 and 16 cells/μl results in a mean neurosphere number generated per well that is proportional to cell density (figure 5.1B); however, the large standard deviations suggest an inconsistent neurosphere number as a result of high-density plating. An indication of the amount of neurospheres available for subsequent processing or passaging is also provided. However, the same analyses shows that low-density plating can result in a non-optimal number of neurospheres (under 30).

Neurospherogenic efficiency was calculated as the percentage of plated cells that formed a neurosphere (figure 5.1C). No observable trend in neurospherogenic efficiency was apparent between the different conditions after 5 days in culture; however cells plated at 7 or 10 cells/μl showed a lower standard deviation from the mean than those cells plated at higher densities. The total number of cells at the end of culture was divided by the total number of neurospheres for each condition, giving a measure of the mean number of cells per sphere, and thus size (figure 5.1D). When these values were plotted, no definitive trend was observed; however, high-density plating was, once more, shown to lead to large standard deviations from the mean, and thus a larger range of neurosphere size.

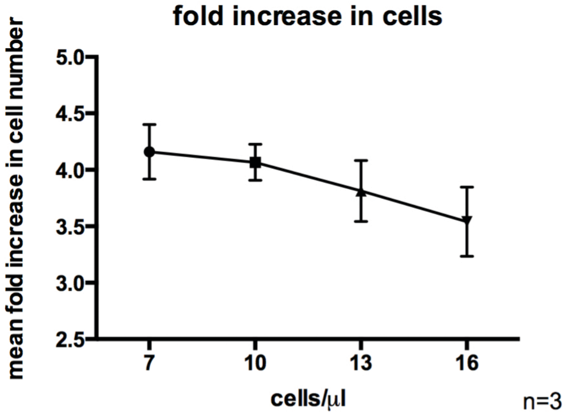
Taken together these results support low-density plating of cells as a method to generate consistent numbers of neurospheres in the hypothalamus that are of a comparable size, and that are generated with consistent efficiency. Observation of the neurospheres over longer culture periods supports this idea, as it suggests that the number of neurospheres decreases as they aggregate and fuse together; therefore the large standard deviation present at high densities is likely an early indication of this process. These data thus confirm low densities as more suitable for clonal density propagation, and in particularly 10 cells/μl as an ideal number, providing sufficient neurosphere numbers for passaging. This density is consistent with the established protocol for the generation of neurospheres from the SVZ and SGZ, and will be the standard density used for all subsequent experiments.

## Figure 5.1: Optimal cell density for neurosphere assay

Dissociated cells from established tertiary (3°) neurospheres are plated at 7, 10, 13 and 16 cells per μl in 400μl of neurosphere media and cultured at 37°C, 5% $CO_2$, for 5 days, fed every 2 nights in culture. At the end of the culture period, neurospheres are quantified, dissociated and the total cell number is quantified for each initial density.

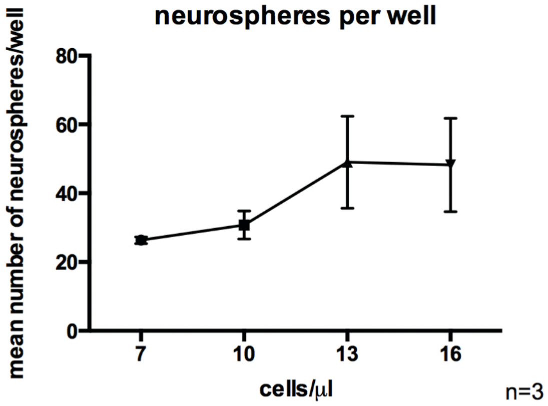
A. Fold increase in cells. The fold change in cell number for each density is calculated and plotted. Error bars represent standard error from mean (SEM). A decreasing trend in fold increase is observed with increasing cell density. The mean, standard deviation from mean (SD) and SEM are presented in tabular form below. Plating at 10 cells/μl results in the lowest SD. Plating at 16 cells/μl results in the highest SD. (*n=3*)
B. Mean number of neurospheres per well. The number of neurospheres per well is quantified for 12 wells (*n=3*) for each density. The mean number of neurospheres is plotted, with an observable trend for increasing neurospheres per well as density increases. Error bars represent SEM. The mean, SD and SEM are presented in tabular form below. Plating at higher densities leads to a high SD, compared to plating cells at lower densities. (*n=3*)
C. Mean neurospherogenic efficiency. The number of neurospheres generated is calculated as a percentage of cells plated, giving the neurospherogenic efficiency as plotted. Error bars represent SEM. No observable trend is observed in neurospherogenic efficiency during these experimental conditions. The mean, SD and SEM are presented in tabular form below. A lower SD from mean neurospherogenic is calculated for low-density plating (7, 10 cells/μl). (*n=3*).
D. Size of neurospheres. The mean number of cells per neurosphere is calculated and plotted for each density. Error bars represent SEM. No distinct trend is observed in cells per sphere with increasing density. The mean, SD and SEM are presented in tabular form below. Plating cells at 7 cells/μl results in the lowest SD, cells plated at 10, 13 and 16 cells/μl all have a comparable SD. (*n=3*)

**A**



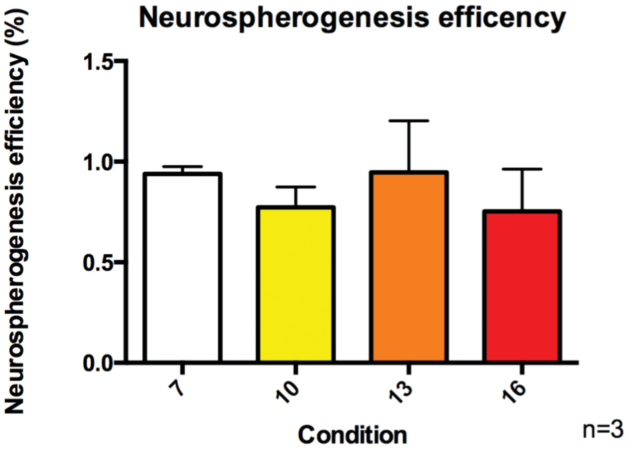
| | 7 | 10 | 13 | 16 |
|---|---|---|---|---|
| Mean | 4.160 | 4.067 | 3.813 | 3.540 |
| Std. Deviation | 0.4187 | 0.2759 | 0.4674 | 0.5311 |
| Std. Error of Mean | 0.2417 | 0.1593 | 0.2698 | 0.3066 |

**B**



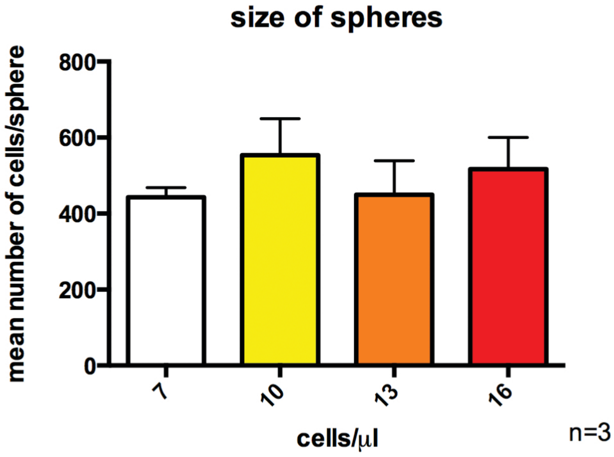
| | 7 | 10 | 13 | 16 |
|---|---|---|---|---|
| Mean | 26.33 | 30.77 | 49.00 | 48.20 |
| Std. Deviation | 1.686 | 7.051 | 23.11 | 23.46 |
| Std. Error of Mean | 0.9735 | 4.071 | 13.34 | 13.54 |

**C**



| | 7 | 10 | 13 | 16 |
|---|---|---|---|---|
| Mean | 0.9400 | 0.7733 | 0.9467 | 0.7533 |
| Std. Deviation | 0.06083 | 0.1750 | 0.4441 | 0.3636 |
| Std. Error of Mean | 0.03512 | 0.1011 | 0.2564 | 0.2099 |

**D**



| | 7 | 10 | 13 | 16 |
|---|---|---|---|---|
| Mean | 443.1 | 553.6 | 449.9 | 516.7 |
| Std. Deviation | 44.25 | 166.7 | 154.8 | 144.3 |
| Std. Error of Mean | 25.55 | 96.26 | 89.37 | 83.31 |

### 5.2.2: Neurospherogenic characteristics of tanycytes

Our characterisation of the tuberal hypothalamus with progenitor markers, combined with the lineage-tracing data, provide evidence of heterogeneity between tanycyte subtypes with respect to progenitor status. To confirm and extend these observations, the in-vitro neurosphere assay was used in order to directly assay the neurospherogenic potential of different hypothalamic regions (figure 5.2: note the data shown in 5.2 A, B was produced by S.Robins), and to elucidate the tanycytes' contribution to neurosphere formation (figure 5.3).

The hypothalamus was dissected into anterior, central (tuberal) and posterior regions, the tissue was dissociated and cultured for 10 days in order to quantify the number of primary and secondary neurospheres that could be generated from each respective region (figure 5.2A). Regions that are known to be rich in tanycytes, the tuberal and posterior hypothalamus, generated neurospheres that could be passaged, while the anterior region generated few primary neurospheres. The tuberal region of the hypothalamus generated robust numbers of both primary and secondary neurospheres compared to anterior and posterior regions. The neurospherogenic potential of different tanycyte subregions was then analysed by careful subdissection based on location, and the ability to form primary neurospheres was quantified (figure 5.2B). Beta-tanycyte-rich regions were unable to generate primary neurospheres, while ependymocyte-rich regions generated few numbers. In contrast, alpha-tanycyte-rich regions generated robust numbers of neurospheres.

To further investigate the neurogenic potential within the different alpha-tanycyte subpopulations, I performed careful sub-dissections of tanycyte regions based on location. The accuracy of the sub-dissections was subsequently confirmed by Gfap expression in sister explants (figure 5.2Ci). Ventral alpha2-tanycytes do not express Gfap, dorsal alpha2-tanycytes express Gfap on long processes, and alpha1-tanycytes express Gfap on short processes. The passaging ability of these regions was subsequently quantified up to passage 8 (8°) (figure 5.2Cii). Ventral alpha2-tanycyte regions

## Figure 5.2: Neurospherogenic potential is dependent on tanycyte region

The adult hypothalamus is subdissected accordingly, and dissociated cells are plated at 10 cells/μl in neurosphere media and cultured at 37°C for 10 days, feeding every 2 nights in culture.

A. The whole hypothalamus is dissected into anterior (green), tuberal (purple) and posterior (red) regions. The number of primary and secondary neurospheres is quantified per well for each region. Error bars represent SEM. Tuberal regions generate robust numbers of primary and secondary neurospheres. Anterior regions generate few primary neurospheres that cannot be passaged to secondary spheres. Posterior regions generate few neurospheres that can be passaged to secondary spheres. (Sarah Robins performed these experiments and analyses, images are adapted from Robins *et al*. (2013) with permission: Ψ)

B. The tuberal hypothalamus is subdissected into beta-tanycyte (red), ventral alpha2-tanycyte (blue), dorsal alpha2-tanycte/alpha-1 tanycyte (purple) and ependymocyte (yellow) rich regions. The number of primary neurospheres generated from each region is quantified and plotted. Error bars represent SEM. Beta-tanycyte regions do not generate primary neurospheres. Alpha-tanycyte regions generate robust numbers of neurospheres. Ependymocyte regions generate few primary neurospheres. Ψ

C.
   i. Further subdissections of the adult tuberal hypothalamus into ventral alpha2-tanycyte (blue), dorsal alpha2-tanycyte (green) and alpha1-tanycyte (purple) rich regions are performed based on location, accuracy of subdissection is confirmed by Gfap-expression in sister explants.
   ii. Neurospheres derived from the corresponding regions are quantified per well. Error bars represent SEM. Ventral alpha2-tanycyte regions generate robust numbers of primary and secondary neurospheres, which subsequently decrease and cannot be passaged beyond the 4th passage (4°). Alpha1-tanycyte

regions generate low numbers of neurospheres that cannot be passaged beyond 7°. Dorsal alpha2-tanycyte regions generate robust numbers of neurospheres, which can be passaged beyond 8° (Up to passage 25, latest time point analysed).

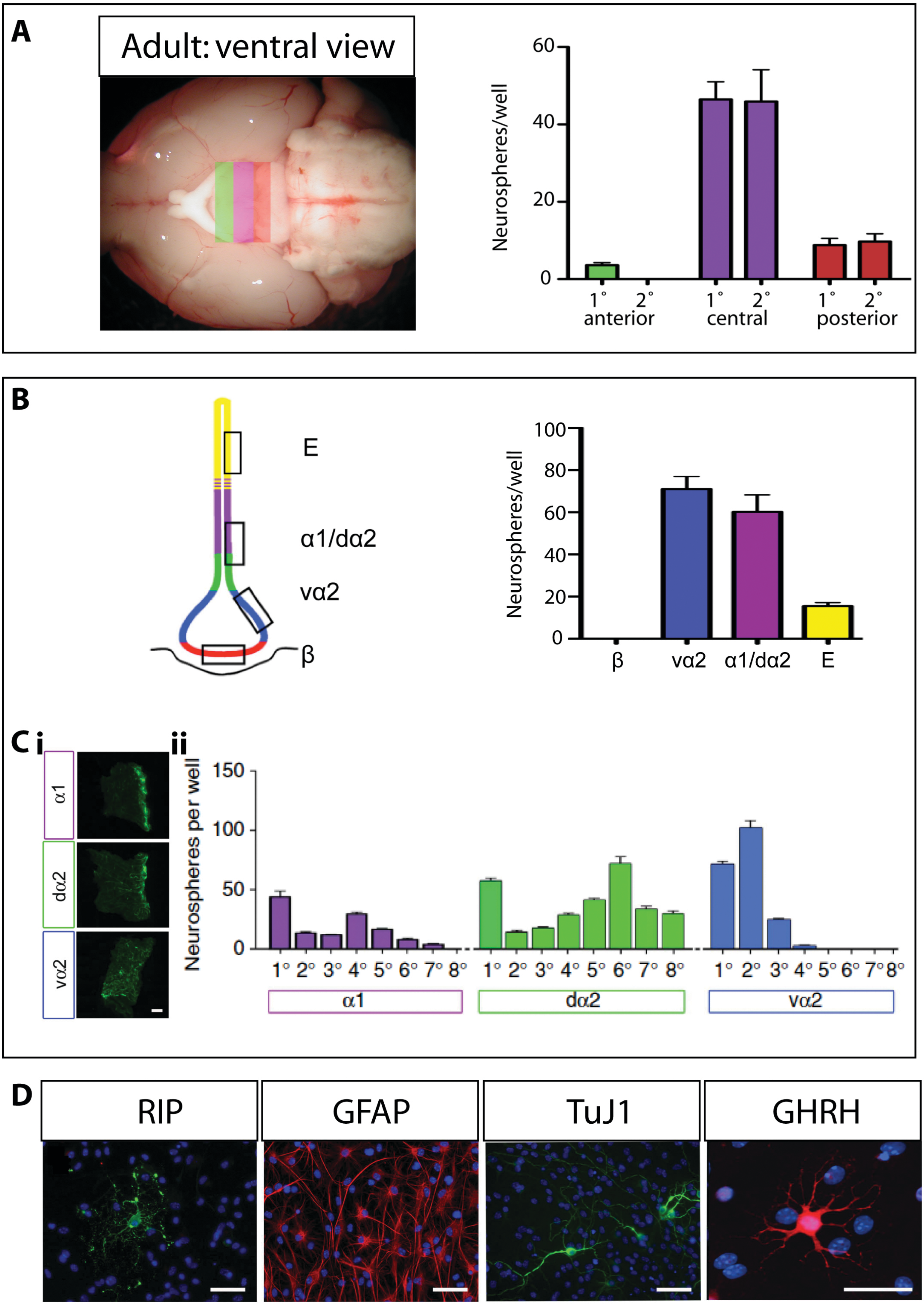
D. Removal of growth-factors leads to hypothalamic neurospheres differentiating into neural lineages. Anti-RIP is used to distinguish oligodendrocyte formation, anti-Gfap marks astrocytes and anti-TuJ1 marks the neuronal lineage. Anti-GHRH (growth-hormone-releasing hormone) is used to identify hypothalamic specific, and physiologically relevant, neuronal differentiation. Scale bar represents 20μm.

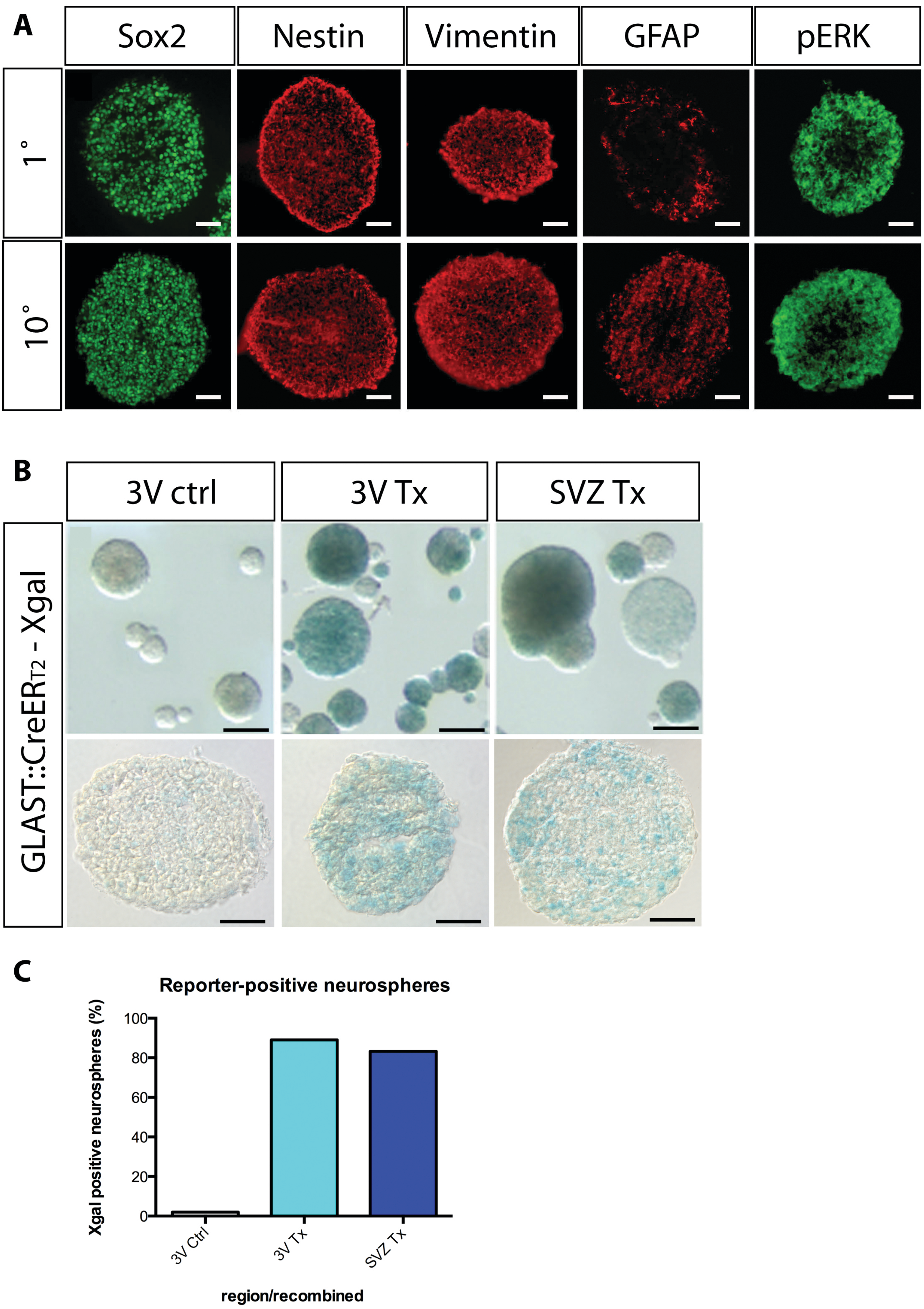
## Figure 5.3: Tanycytes are neurospherogenic

A. Primary and 10° neurospheres are fixed and labeled with known tanycytic markers Sox2, Nestin, Vimentin, Gfap; and a marker for growth-factor activity, phosphorylated-extracellular signal-related kinase (pERK). Strong signal for Sox2, Nestin, Vimentin and pERK is detected at 1° and maintained at 10°. Weak Gfap staining is observed at 1°, which increases by 10°. Scale bars represent 50μm. Ψ

B. Adult Glast::CreER$^{T2}$ are injected with tamoxifen (Tx) to induce recombination in *LacZ* reporter mice, or with vehicle alone. Neurospheres are generated from the non-recombined hypothalamus (3V-control), the recombined hypothalamus (3V-Tx) or the recombined SVZ of the lateral ventricles as a positive control (SVZ-Tx). 6° neurospheres were fixed and stained with Xgal wholemount (top panel) or on 15μm sections (bottom panel). Xgal does not stain 3V-control neurospheres, while strong Xgal signal is detected in 3V-Tx and SVX-Tx neurospheres. Scale bars represent 200μm (top panel); 50μm (bottom panel).

C. Number of Xgal-positive 6° neurospheres is quantified for 3V-Ctrl, 3V-Tx and SVZ-Tx, and the proportion of total neurospheres is plotted. The majority of 3V-Tx (89.1%) and SVZ-Tx (83.3%) neurospheres are reporter-positive, a small minority of 3V control neurospheres are stained with Xgal (2%). (*n=1*)


A
Adult: ventral view
Neurospheres/well
0
20
40
60
1°
2°
anterior
1°
2°
central
1°
2°
posterior
B
E
α1/dα2
vα2
β
Neurospheres/well
0
20
40
60
80
100
β
vα2
α1/dα2
E
C
i
α1
dα2
vα2
ii
Neurospheres per well
0
50
100
150
1° 2° 3° 4° 5° 6° 7° 8°
α1
1° 2° 3° 4° 5° 6° 7° 8°
dα2
1° 2° 3° 4° 5° 6° 7° 8°
vα2
D
RIP
GFAP
TuJ1
GHRH


A
Sox2
Nestin
Vimentin
GFAP
pERK
1°
10°
B
3V ctrl
3V Tx
SVZ Tx
GLAST::CreER$_{T2}$ - Xgal
C
Reporter-positive neurospheres
Xgal positive neurospheres (%)
100
80
60
40
20
0
3V Ctrl
3V Tx
SVZ Tx
region/recombined

generated large numbers of neurospheres initially, but these numbers decreased with successive passaging, and could not be passaged beyond 4°. Similarly, alpha1-tanycyte regions could not be passaged beyond 7°. Conversely, dorsal alpha2-tanycyte regions generated neurospheres that could be passaged successfully beyond 8°. These data indicate that heterogeneity exists between tanycyte subtype regions with respect to their neurospherogenic potential, an established in-vitro assay for neural stem-like features. Furthermore, neurospheres are able to differentiate into oligodendrocytes, astrocytes and neurons, confirming their multipotency; the generation of a hypothalamic specific neuronal cell type, a growth-hormone-releasing hormone-positive neuron (figure 5.2D) supports the notion that neurogenesis from the hypothalamic niche has a functional relevance.

Although the above results demonstrate that cells within different tanycyte-rich regions have distinct neurospherogenic potential, these data do not confirm tanycytes as the neurospherogenic cell type: it is formally possible that a second cell type exists within the tanycyte-rich VZ and is the neurospherogenic cell. In order to begin to address whether tanycytes generate neurospheres, antibodies against known tanycyte markers: Sox2, Nestin, Vimentin and Gfap, were used to identify whether hypothalamic neurospheres share this marker expression (figure 5.3A). Primary neurospheres expressed Sox2, Nestin and Vimentin, and showed weak expression of Gfap. All four markers were maintained in 10° neurospheres, with an increase in Gfap expression. These data support tanycytes as neurospherogenic, and the change in Gfap expression suggests neurospheres that can be serially passaged in culture are derived from Gfap-positive alpha-tanycytes.

To further confirm the alpha-tanycyte origin of neurospheres, Glast::CreER$^{T2}$ mice were injected with tamoxifen to induce recombination, or vehicle alone as a negative control. Recombination of the Glast::CreER$^{T2}$ mice leads to reporter activity in alpha-tanycytes specifically (chapter 4). The hypothalamus was dissected from recombined and non-recombined mice; in addition, the SVZ of the lateral ventricles was dissected from recombined mice as a positive control.

Tissue was dissociated and maintained in neurosphere culture until 6° neurospheres were generated. These neurospheres were fixed and stained with X-gal in order to detect beta-galactosidase activity and thus *lacZ* reporter expression, in both wholemount neurospheres and 15μm sections (figure 5.3B). Neurospheres generated from the recombined hypothalamus stained blue in response to X-gal treatment, compared to those from the non-recombined mice, supporting reporter activity in recombined neurospheres. The number of Xgal stained neurospheres was quantified in wholemount neurospheres, and the number was plotted as a proportion of all neurospheres in each condition (figure 5.3C). Hypothalamic neurospheres showed a comparable number of reporter-positive neurospheres to the positive control, the SVZ, supporting Glast::CreER$^{T2}$ positive tanycytes as neurospherogenic.

In conclusion, these data provide strong evidence of heterogeneity relative to tanycyte subregion. They demonstrate that regions containing beta-tanycytes are not neurospherogenic. By contrast, dorsal alpha2-tanycyte-rich regions generate robust numbers, which can be successfully passaged long-term. Neurospheres share markers with alpha-tanycytes. Finally, reporter-positive neurospheres are generated from Glast::CreER$^{T2}$ recombined mice.

### 5.2.3: Hypothalamic neurospheres require Fgf-signalling and are responsive to local Fgf ligands

The in-vivo infusion regime (chapter 4) provided the first evidence that cells within the hypothalamic niche are responsive to Fgfs. Added to this, analyses of neurospheres revealed their expression of phosphorylated extracellular-signal related kinase (pERK), an effector of Fgf signalling, (figure 5.3Ai). Together, these analyses suggested that hypothalamic stem and/or progenitor cells are Fgf-responsive. Within the lab, we therefore set out to examine the expression of *Fgfs* within the hypothalamus and to exploit the neurosphere assay to examine the response of isolated hypothalamic cells to Fgfs. The initial studies were performed by S. Robins (section 1.1.6).

## Figure 5.4: Hypothalamic neurospheres are Fgf dependent

A. Sarah Robins performed these experiments and analyses, images are adapted from Robins *et al*. (2013) Ψ.

  i. Neurospheres are generated in neurosphere media contain EGF and FGF2 (beige), FGF2 alone (light-blue), EGF alone (dark-blue) or without growth-factors (black). Neurospheres per well are quantified and plotted. Spheres are dissociated and passaging ability is determined. Error bars represent SEM. Robust neurosphere numbers are generated with both growth-factors that can be passaged beyond 5°. In the absence of EGF, primary neurospheres are generated which cannot be passaged beyond 2°. In the absence of FGF2, low numbers of neurospheres are generated, which decrease to passage 5. In the absence of both EGF and FGF2, few neurospheres are generated that cannot be passaged beyond 1°.

  ii. Dissociated hypothalamic tissue is cultured in neurosphere media containing FGF2 and EGF; FGF2, EGF and FGF inhibitor (SU5402); EGF alone; or EGF and SU5402. Neurospheres per well is quantified. Error bars represent SEM. No primary neurospheres are generated with FGF inhibitor. Comparable numbers of primary neurospheres are generated in media containing FGF2 and EGF or EGF alone.

  iii. RT-PCR is performed on neurospheres to detect mRNA for *Fgf10* and *Fgf18*. Both *Fgf10* and *Fgf18* mRNA expression is detected.

B. 4° neurospheres are generated in media containing EGF alone (blue); EGF and FGF2 (orange); EGF and FGF10 (red); EGF and FGF18 (pink); EGF, FGF10 and FGF18 (yellow). Cells are cultured at 37°C for 7 days, fed every 2 nights.

  i. Mean number of neurospheres per well is quantified and plotted. Error bars represent SEM. A significant increase in neurosphere number is calculated in media containing FGF10 compared to FGF2 ($p<0.05$ =*). A trend of increase neurosphere generation is observed in media containing EGF and FGF10 compared to other media. ($n=12$).

ii. Mean number of cells per sphere is quantified and plotted. Error bars represent SEM. Media containing EGF and FGF2 results in a statistically significant increase in cells per sphere above all other media ($p<0.05$ =*). Media containing FGF18 leads to a significant increase in cells per sphere, above media containing EGF alone or EGF and FGF10. ($n=12$).

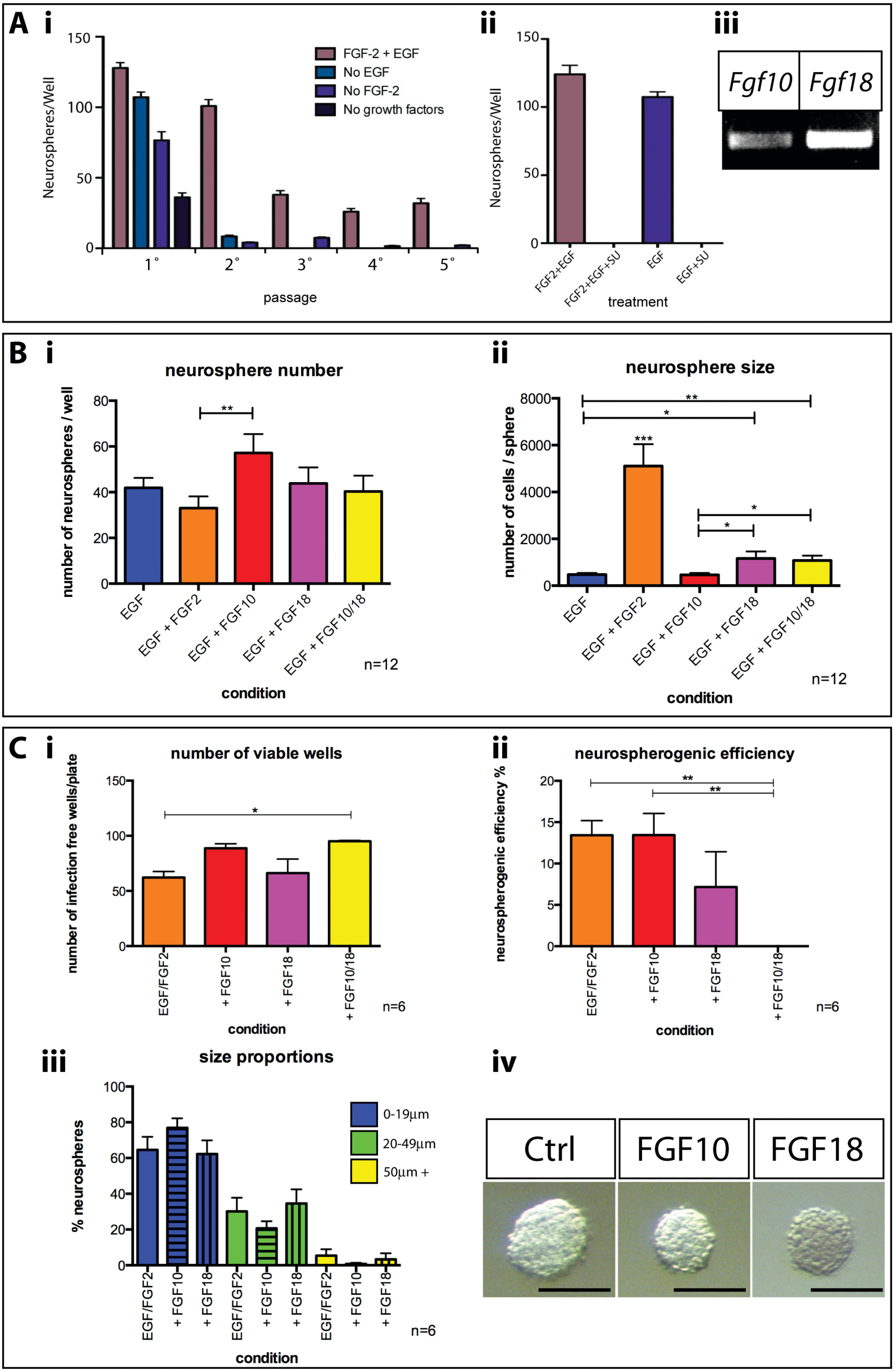
C. 4° neurospheres are dissociated and plated as single cells in 96 well plates ($n=6$) to determine whether clonal spheres can be generated. Cells are cultured in media containing EGF and FGF2 as control (orange), plus FGF10 (red), FGF18 (pink) or FGF10 and FGF18 (yellow). Cells are cultured at 37°C for 10days, fed every 2 nights.

i. Total number of neurospheres per 96 well plate is quantified and plotted for each media. Error bars represent SEM. More neurospheres are quantified in media containing FGF10 compared to all other media, statistically significant compared to media containing EGF, FGF2, FGF10 and FGF18 ($p<0.05$ =*). No neurospheres are detected in media containing all growth factors.

ii. Neurospherogenic efficiency is calculated as the percentage of infection-free wells in which neurospheres formed. Error bars represent SEM. Media containing EGF/FGF2 and EGF/FGF2/FGF10 result in the highest percentage survival, statistically significant compared to media containing EGF/FGF2/FGF10/FGF18 ($p<0.05$ =*).

iii. A graticule was used to measure the diameter of clonal neurospheres into 3 categories: small (0-19μm), medium (20-49μm), large (50μm+). The percentage of clonal neurospheres that are within those categories is plotted for each media in which neurospheres are detected. Error bars represent SEM. Media containing EGF/FGF2/FGF10 results in a trend for more small neurospheres and fewer medium and large neurospheres compared to other media. No difference is detected between media containing EGF/FGF2 and EGF/FGF2/FGF18.

iv. Light microscope images of clonal neurospheres generated in media EGF/FGF2, +FGF10 and +FGF18. Scale represents 20μm.


A
i
150
100
50
0
Neurospheres/Well
FGF-2 + EGF
No EGF
No FGF-2
No growth factors
1˚
2˚
3˚
4˚
5˚
passage
ii
150
100
50
0
Neurospheres/Well
FGF2+EGF
FGF2+EGF+SU
EGF
EGF+SU
treatment
iii
Fgf10
Fgf18
B
i
neurosphere number
80
60
40
20
0
number of neurospheres / well
**
EGF
EGF + FGF2
EGF + FGF10
EGF + FGF18
EGF + FGF10/18
n=12
condition
ii
neurosphere size
8000
6000
4000
2000
0
number of cells / sphere
**
*
***
*
*
EGF
EGF + FGF2
EGF + FGF10
EGF + FGF18
EGF + FGF10/18
n=12
condition
C
i
number of viable wells
150
100
50
0
number of infection free wells/plate
*
EGF/FGF2
+ FGF10
+ FGF18
+ FGF10/18
n=6
condition
ii
neurospherogenic efficiency
20
15
10
5
0
neurospherogenic efficiency %
**
**
EGF/FGF2
+ FGF10
+ FGF18
+ FGF10/18
n=6
condition
iii
size proportions
100
80
60
40
20
0
% neurospheres
0-19µm
20-49µm
50µm +
EGF/FGF2
+ FGF10
+ FGF18
EGF/FGF2
+ FGF10
+ FGF18
EGF/FGF2
+ FGF10
+ FGF18
n=6
condition
iv
Ctrl
FGF10
FGF18

To begin to address the cellular response to Fgfs, hypothalamic tissue was dissociated and cultured in media containing EGF/FGF2, FGF2, EGF or no growth-factors (work performed by S. Robins). The potential to generate primary neurospheres that could be passaged was quantified for each media. Media containing EGF/FGF2 resulted in robust numbers of neurospheres that could be passaged. In the absence of growth-factors, primary neurospheres were formed but they could not be passaged, suggesting EGF and/or FGF2 is required for passaging success. In accordance, neurospheres generated in the absence of EGF could not be passaged beyond 2°, while in the absence of FGF2 very few numbers of neurospheres could be passaged.

To determine whether Fgf signalling was required for neurosphere formation, the Fgf-receptor inhibitor, SU5402, was added to neurosphere media containing EGF/FGF2 or EGF (figure 5.4Aii: work performed by S. Robins). In the absence of SU5402, no neurospheres were generated, providing evidence that neurospherogenesis is dependent upon Fgf signalling. Furthermore, neurospheres were generated in media containing EGF alone, while addition of SU5402 inhibited this, suggesting neurospheres are not reliant upon an initial, external source of FGF2. To assess whether neurospheres generate Fgfs independently, RT-PCR was performed on homogenised neurospheres to detect mRNA expression of endogenous Fgfs: *Fgf10* and *Fgf18* (figure 5.4iii). Neurospheres express detectable levels of *Fgf10* mRNA and high levels of *Fgf18* mRNA, i.e. *Fgfs* that are also expressed in restricted tanycyte regions. These data indicate Fgf10 and Fgf18 are synthesised by neurospherogenic cells, and may compensate for FGF2 in neurosphere culture.

Building on these studies, I set out to examine the response of neurospheres to FGF10 and FGF18, i.e. the Fgfs that overlap alpha 2 tanycytes in-vivo. Neurosphere were cultured in media containing EGF alone, or with the addition of FGF10, FGF18, FGF10/FGF18, or for comparison, FGF2 (figure 5.4B). The addition of FGF10 resulted in an observable increase in neurosphere number compared to the other conditions, significantly more than with the addition of FGF2 (figure 5.4Bi). However, neurospheres cultured in the presence of FGF2 were significantly larger in size than all other conditions, i.e. contained significantly more cells (figure 5.4Bii). It seems likely, then, that in the presence of FGF2, many small neurospheres aggregated to generate few large neurospheres, accounting for the low number of neurospheres. Yet, media containing FGF18 have significantly larger neurospheres, as measured by number of cells, compared to EGF alone and EGF/FGF10. These data indicate FGF10 can increase neurosphere number, while FGF18 can increase neurosphere size.

In order to further investigate the difference in the neurospherogenic characteristics of the endogenous Fgfs, single cells were plated at the clonal level, not clonal density. Neurospherogenesis of clonal spheres from the SVZ indicates isolated single cells do not grow as efficiently as cells plated at clonal density, due to the paracrine factors from other cells providing a conditioned media to encourage growth. Therefore, in this experiment EGF is added with FGF2 in control media in order to support neurosphere formation, while avoiding the caveats of neurosphere aggregation and fusion in prolonged clonal density culture (figure 5.B).

Single cells were sorted into each well of six 96-well plates for each condition: EGF/FGF2 as control, with the addition of FGF10, FGF18 or FGF10/18 (figure 5.4C). As infection was present in a number of wells, the total number of infection free wells was quantified by careful observation (figure 5.4Ci). Neurospherogenic efficiency was calculated as the percentage of infection free wells in which a neurosphere formed (figure 5.4Cii). The size of neurospheres in each condition was measured using a graticule and assigned to one of three groups: small (0-19μm), medium (20-49μm) and large (50+μm) (figure 5.4Ciii). Light microscope images of clonal neurospheres are shown in figure 5.4Civ. Infection free wells were observed in all conditions, however a discrepancy is found between conditions. EGF/FGF2 control media suffered the most infection with a mean number of 62.2 (±5.6) viable wells, followed by FGF18 media at 66.2 (±12.8), FGF10 media at 88.7 (±4.2) and FGF10/18 media at 95.0 (±0.8) wells. Calculating neurospherogenic efficiency reveals no significant differences between control media and media containing added FGF10 or FGF18, however a decrease in mean percentage efficiency is observed with the addition of

FGF18. Furthermore, the addition of all four growth-factors results in no neurosphere formation.

The size proportions of neurospheres generated in FGF18 media is comparable to EGF/FGF2 control spheres, while in contrast, more small neurospheres and fewer/medium large neurospheres are generated in FGF10 media. These results are consistent with previous data, supporting FGF10 as a survival factor that leads to small neurospheres. However, the difference in neurospherogenic efficiency between control media and the addition of FGF18 suggests these three growth-factors together can impede neurosphere formation.

Together these data reveal hypothalamic neurospherogenic cells as Fgf-dependent, and identify endogenously expressed Fgfs. Addition of the endogenous Fgfs to neurosphere media indicates that FGF10 increases neurosphere number at the expense of size, suggesting a survival role; while FGF18 can increase neurosphere size at the expense of number, suggesting a proliferative role in this in-vitro culture assay. Furthermore, the generation of clonal neurospheres from single cells supports a neural stem cell characteristic that cannot be defined by clonal density analysis alone.

### 5.2.4: The neurosphere assay as a tool to assess the physiological relevance of the hypothalamic niche

While developmental signals, such as Fgfs, are classically defined regulators of proliferation, emerging evidence supports neural progenitor cells as responsive to physiological stimulation and neuronal activation (Muth-Kohne *et al.*, 2010; Song *et al.*, 2012). An important question is whether neural progenitor cells exhibit voltage- and/or ligand-gated channels themselves, a characterising feature of functional neurons. This is of significance, as it could indicate that proliferation and/or differentiation of progenitors is directly responsive to physiological activation/inhibition of circuitry. Indeed, recent studies have implicated excitatory neurotransmission in increasing neurogenesis (Brazel *et al.*, 2005b; Nacher and McEwen, 2006; Xiao *et al.*, 2013). Glutamate is the main excitatory neurotransmitter throughout the central nervous system, and thus the NMDA receptor, an ionotropic glutamate receptor, is crucial to neuronal communication. Here, NMDA is added to neurosphere media containing EGF/FGF2 in order to investigate whether neurospherogenic cells are responsive to excitatory glutamate signalling (figure 5.5).

The number of neurospheres per well was quantified and the average for the three conditions is plotted (figure 5.5Ai). The number of neurospheres formed is calculated as a percentage of the cells plated and plotted as the neurospherogenic efficiency (figure 5.5Aii). A haemocytometer is used to quantify the number of cells present after dissociation of neurospheres in order to calculate the average number of cells per sphere (figure 5.5Aiii) and the fold increase in cells (figure 5.5Aiv), providing a measure of neurosphere size and proliferation in each condition. Addition of 10μM or 100μM NMDA resulted in a significant increase in neurosphere number above the control, containing EGF/FGF2 alone (figure 5.5Ai). The comparatively low number of neurospheres generated from an equal number of cells (neurospherogenic efficiency), as well as the larger neurosphere size in 100μM NMDA media compared to 10μM NMDA media, provides evidence that NMDA incrementally increases proliferation, resulting in neurosphere fusion at high concentrations (figure 5.5A). Neurospherogenic cells are therefore responsive to the excitatory neurotransmitter glutamate, through NMDA-receptor activation.

To investigate whether NMDA exerts the neurospherogenic effect at the level of the neural stem cell or a potential intermediate progenitor, 10μM NMDA was added to neurosphere media for a single culture period, and after passaging, the dissociated cells were placed into control media (figure 5.5B). If NMDA increases the proliferation of transit-amplifying progenitors, one could expect to see a decrease in neurospherogenesis below the control in the subsequent passage, as progenitor derived neurospheres may fail to propagate in comparison to control neurospheres. In line with the previous experiment, addition of 10μM NMDA increases neurosphere number compared to control media at passage one, however a comparable number of neurospheres is

## Figure 5.5: Neurospherogenic efficiency increases in response to NMDA

A. 4° neurospheres are generated in media containing EGF/FGF2 as a control, plus 10μM NMDA or 100μM N-methyl-D-aspartate (NMDA). Neurospheres are quantified per well, cells are then dissociated and quantified. Error bars represent SEM.

   i. Mean neurosphere number per well. Addition of 10μm NMDA results in a significant increase in mean neurosphere number, above media containing EGF/FGF2 and EGF/FGF2/100μm-NMDA (*p<0.05 =**). (*n=72*).

   ii. Mean neurospherogenic efficiency. The number of neurospheres generated is plotted as a percentage of cells plated to give neurospherogenic efficiency. A significant increase in neurospherogenic efficiency is calculated for media containing 10μM NMDA compared to EGF/FGF2 alone (*p<0.05 =**). Increased concentration of NMDA in the media results in a lower neurospherogenic efficiency compared to 10μM, although not statistically significant. (*n=3*).

   iii. The mean number of cells per sphere is calculated and plotted for each media. No significant difference is calculated between conditions. The mean number of cells per sphere is highest in media containing 100μM NMDA. (*n=3*)

   iv. The mean fold increase in cell number is calculated and plotted for each condition. A trend of increasing fold change in cell number is observed for increasing concentrations of NMDA (*n=3*).

B. 5° neurospheres are generated in media containing EGF/FGF2 alone (control) or with 10μM NMDA; passage 1. Neurospheres are quantified, dissociated and plated at 10 cells/μl in control media (EGF/FGF2) to generate 6° neurospheres; passage 2. Error bars represent SEM.

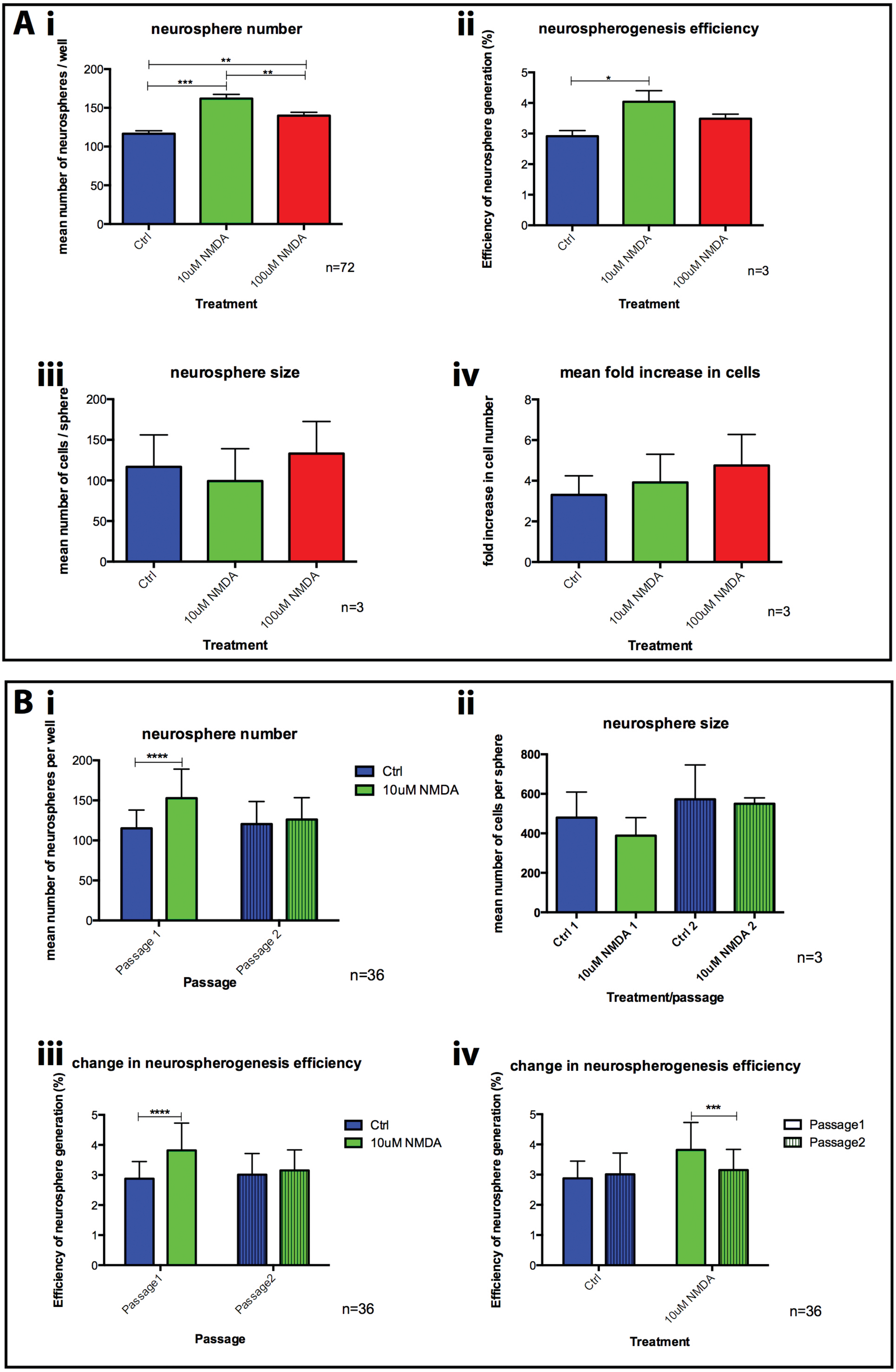
   i. Mean neurosphere number per well is plotted for each media at passage 1 and passage 2. A significant increase in neurosphere number is calculated in media containing 10μM NMDA (*p<0.05 =**)

compared to EGF/FGF2 alone. No statistical significance is observed at passage 2. ($n=36$).

ii. Mean number of cells per sphere is calculated and plotted for each condition at each passage. No statistical significance is calculated between conditions. A trend for fewer cells per sphere is observed in media containing 10μM NMDA. ($n=3$).

iii. Neurospherogenic efficiency at each passage is plotted according to condition. A statistically significant increase in neurospherogenic efficiency is calculated between control and NMDA media at passage 1 ($p<0.05$ *=**), while no difference is observed at passage 2. ($n=36$)

iv. Neurospherogenic efficiency for each condition is plotted according to passage number. A statistically significant decrease in neurospherogenic efficiency is calculated for media that contained NMDA at passage 1 compared to passage 2 ($p<0.05$ *=**). No difference is observed in neurospherogenic efficiency between passage 1 and passage 2 in control media. ($n=36$).


A
i
neurosphere number
mean number of neurospheres / well
200
150
100
50
0
***
**
**
Ctrl
10uM NMDA
100uM NMDA
n=72
Treatment
ii
neurospherogenesis efficiency
Efficiency of neurosphere generation (%)
5
4
3
2
1
0
*
Ctrl
10uM NMDA
100uM NMDA
n=3
Treatment
iii
neurosphere size
mean number of cells / sphere
200
150
100
50
0
Ctrl
10uM NMDA
100uM NMDA
n=3
Treatment
iv
mean fold increase in cells
fold increase in cell number
8
6
4
2
0
Ctrl
10uM NMDA
100uM NMDA
n=3
Treatment
B
i
neurosphere number
mean number of neurospheres per well
200
150
100
50
0
****
Ctrl
10uM NMDA
Passage 1
Passage 2
Passage
n=36
ii
neurosphere size
mean number of cells per sphere
800
600
400
200
0
Ctrl 1
10uM NMDA 1
Ctrl 2
10uM NMDA 2
n=3
Treatment/passage
iii
change in neurospherogenesis efficiency
Efficiency of neurosphere generation (%)
5
4
3
2
1
0
****
Ctrl
10uM NMDA
Passage1
Passage2
n=36
Passage
iv
change in neurospherogenesis efficiency
Efficiency of neurosphere generation (%)
5
4
3
2
1
0
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Passage1
Passage2
Ctrl
10uM NMDA
n=36
Treatment

generated when cells that had previously seen NMDA were cultured in control media (figure 5.5Bi,iii). These data suggest NMDA acts upon the same cell-type as EGF and FGF2, as no difference is observed after passaging between control and NMDA group, implying the two populations are indistinguishable with respect to neural stem cell and proliferative progenitor ratios.

The neurosphere assay is shown here to be a valuable tool in confirming the in-vivo analysis of alpha-tanycytes as a neural stem/progenitor population, and further characterise the FGF2-responsive niche as having dependency upon Fgf-signalling for proliferation in-vitro. The neurosphere assay has also been used to evaluate whether neurospherogenic cells, alpha-tanycytes, are responsive to a physiological stimulus, such as NMDA receptor activation that would be induced in-vivo by the excitatory neurotransmitter, glutamate. The increase in neurospherogenic efficiency in response to NMDA supports the hypothalamic niche, and indeed alpha-tanycytes, as proliferative in response to neuronal circuitry activation.

## 5.3: Discussion

My previous data provides compelling evidence for a neural stem cell population within alpha2-tanycytes of the adult hypothalamus. In addition, I observe heterogeneity in tanycyte subdomains with respect to progenitor status, and identify alpha2-tanycytes as responsive to Fgf signaling. Here I complemented and extended these observations, using the ex vivo neurosphere assay to investigate the self-renewal potential of different hypothalamic regions in-vitro, and to elucidate their response to Fgfs and physiological stimuli. In order to generate data comparable to other niches and hypothalamic studies, a standard neurosphere protocol was adapted from Giachino *et al.* (2009), and cells were grown at a clonal density of 10 cells/μl (figure 5.1).

### 5.3.1: Alpha-tanycytes are neurospherogenic

My lineage-tracing analysis supports alpha-tanycytes as self-renewing, multipotent stem cells in the adult hypothalamus. In addition, the heterogeneity of tanycytes with respect to progenitor characteristics is suggested by observations that Glast::CreER$^{T2}$ tanycytes can generate other tanycyte subtypes. Here, I addressed whether tanycyte subtypes are neurospherogenic, and have particular neurospherogenic features.

Initial observations confirmed that neurospherogenic potential is confined to the tuberal and posterior regions of the hypothalamus, areas rich in tanycytes (figure 5.2A). Further precise subdissection according to tanycyte region reveals that beta-tanycyte regions are unable to form neurospheres (figure 5.2B), while in stark contrast, dorsal alpha2-tanycyte regions can generate robust numbers of neurospheres, that can be serially-passaged long term (figure 5.2C). This in-vitro assay also reveals heterogeneity in progenitor characteristics between ventral alpha2-tanycyte and dorsal alpha2-tanycyte domains (separated on the basis of Gfap expression), revealing that they differ in their passaging potential. Traditionally, multiple rounds of neurosphere dissociation and cell resuspension at clonal density is used to indicate self-renewal capacity of neural stem cells, while an inability to undergo serial passages is considered evidence of neurospheres that are derived from restricted progenitors (Pastrana *et al.*, 2011). Employing these definitions, I conclude that a neural stem cell population resides in the dorsal alpha2-tanycyte subtype, while ventral alpha2-tanycytes constitute a proliferative progenitor population. The limited neurospherogenic ability of alpha1-tanycyte regions also supports the presence of a restricted progenitor population in this location, while no progenitors or stem cells reside in the adult beta-tanycyte region.

A caveat to this, however, is that, while the dogmatic principals (i.e. that define a cell as stem or progenitor) have been widely used by researchers employing the neurosphere assay, this technique evaluates the potential of cells to behave like stem cells in the absence of their niche and in the presence of saturating levels of exogenous growth-factors. In fact, the traditional view that progenitors cannot be serially passaged has now been refuted (Pastrana *et al.*, 2011). Studies have identified that EGF converts SVZ C-cells, transit-amplifying progenitors, into multipotent B-like cells when in culture (Doetsch *et al.*, 2002). Moreover, additional studies in the SVZ have identified transit amplifying C-cells as highly neurospherogenic, while the potential of quiescent neural stem cells remains unknown and masked by the highly neurospherogenic progenitors (Pastrana *et al.*, 2011). It can therefore be argued that isolation of cells and addition of growth-factors causes culture artefact, resulting in a bias towards progenitor dedifferentiation and self-renewal. In light of these considerations, careful interpretation of neurospherogenic potential of the tanycyte subregions is required. In particular, definitive markers that distinguish between quiescent neural stem cell populations and more restricted progenitors are required.

Thus, from the neurosphere assay alone, it is not possible to confidently confer neural stem cell identity to a particular tanycytic subdomain. However, the heterogeneity of tanycyte subdomains in their neurospherogenic potential does provide in-vitro support to the in-vivo lineage-analysis data, where a spreading of alpha1- and beta1- tanycytes descendants was found from alpha2-tanycytes. Together, these lines of evidence are highly supportive of a dorsal alpha2-tanycyte neural stem cell population, which can give rise to adjacent populations of progenitors. Further verification of this interpretation is provided by analysis of tanycyte markers in primary and serially passaged neurospheres (figure 5.3). The observation that adult hypothalamic neurospheres can generate hypothalamic neurons, identified by GHRH expression, supports a neurospherogenic cell type that has the potential to generate physiologically relevant cell types. In addition, this result extends previous studies, which show embryonically derived neurospheres can differentiate into functional hypothalamic neurons (Sousa-Ferreira *et al.*, 2011), by providing evidence that adult derived neurospheres retain this potential.

Sox2, expressed by heterogenous populations of neurospherogenic cells throughout the CNS (Brazel *et al.*, 2005a), and expressed by tanycytes, is expressed by primary and passaged neurospheres (figure 5.3A). Similarly, expression of the tanycyte and classical neural stem cell markers Nestin and Vimentin, is maintained from primary neurospheres to 10° neurospheres. In contrast, Gfap expression in primary neurospheres is weak compared to serially passaged neurospheres. This could indicate that the majority of hypothalamic primary neurospheres are derived from Gfap-negative, ventral alpha2-tanycytes, which are lost through subsequent passages, while Gfap-positive, dorsal alpha2-tanycytes are maintained to 10° spheres, where they now represent the majority. Alternatively, the addition of exogenous growth-factors could change the gene expression level of Gfap in culture, making it difficult to extrapolate tanycyte identity based on marker expression.

In order to provide an additional line of evidence, which together with previous data could definitively identify alpha-tanycytes as neurospherogenic neural stem cells, neurospheres were derived from Glast::CreER$^{T2}$ positive cells of the hypothalamus (figure 5.3B). X-gal was used to detect *LacZ*-reporter expression in wholemount neurospheres, as active beta-galactosidase breaks X-gal down into galactose and an indole group, which dimerises to form a blue product. Quantifying the number of blue neurospheres therefore provides a measure of the number of spheres which are derived from a Glast::CreER$^{T2}$-positive cell. While these data cannot account for a neurospherogenic cell type within the parenchymal Glast::CreER$^{T2}$-positive population, combined with the lineage-tracing data it supports Glast::CreER$^{T2}$-positive alpha-tanycytes as multipotent, self-renewing neural stem cells, which are neurospherogenic in culture.

An inherent caveat in using X-gal staining to detect beta-galactosidase activity is the potential for breakdown by endogenous galactosidases, thus resulting in non-specific and false positive staining (Sanchez-Ramos *et al.*, 2000). In accordance, minimal numbers of neurospheres were observed to show some blue stain in those generated from the non-recombined hypothalamus. However this small level of non-specific staining cannot account for the large percentage of positive neurospheres in the recombined hypothalamus, and SVZ as a positive control.

In summary, hypothalamic neurospheres are shown to express tanycyte markers and can be derived from Glast::CreER$^{T2}$-positive alpha-tanycytes. These data confirm and extend our in-vivo analyses, identifying distinct neurospherogenic features between tanycyte subtypes and supporting dorsal alpha2-tanycytes as maintaining the potential to behave as neural stem cells in-vitro.

## 5.3.2: Hypothalamic neurospheres are Fgf-dependent

Intracerebroventricular administration of FGF2 induces a proliferative response from alpha2-tanycytes (chapter 4), while previous studies in Placzek lab have identified gene expression of endogenous Fgfs in regions restricted to alpha2-tanycytes (*Fgf18*) and both alpha2- and beta-tanycytes (*Fgf10)* (Robins *et al.*, 2013a). In addition, recent studies identify Fgf10-positive tanycytes as neurogenic in response to dietary changes (Haan *et al.*, 2013). As the neurosphere assay presents a tool to disseminate the role of factors in mediating proliferation at the cellular level, we investigated the role of the Fgf-signalling pathway in enhancing neurospherogenic potential (figure 5.4).

As in the SVZ and the SGZ, EGF was found to be required for serial passaging of neurospheres. Likewise, Fgf was required: addition of SU5402, an Fgf receptor-specific tyrosine-kinase inhibitor, which prevents phosphorylation of the Fgf receptor (FgfR) and subsequent signalling, inhibited primary neurosphere formation. Since, in the absence of exogenous Fgf, low numbers of neurospheres could be passaged to 5°, this suggests that endogenous Fgfs regulate neurosphere formation. This suggests that Fgf is required at the level of the neurospherogenic cell, and not solely for the proliferation of restricted progenitors generated within neurospheres. Furthermore, the contrast in neurosphere number, between no exogenous Fgf and FgfR inhibition, supports the expression of endogenous Fgfs by the neurospheres, a conclusion confirmed by RT-PCR (figure 5.4Aiii). The use of FgfR inhibition increases our understanding of neurospherogenic alpha-tanycytes, from in-vivo infusion studies that provided evidence of their Fgf-responsive nature (chapter 4), to in-vitro studies that support a cellular dependency on Fgf for proliferation and self-renewal. This argument is strengthened by the endogenous expression of *Fgf10* and *Fgf18*, both in the tanycyte niche in-situ and by hypothalamic neurospheres themselves.

Established 4° neurospheres were used to elucidate the role of the endogenous Fgfs. EGF was added as standard as results support its required role in neurospherogenesis, with the addition of FGF2 as a positive control for neurosphere formation. FGF10 and FGF18 were added at the same concentration as FGF2, and FGF10 and FGF18 were also added together to identify any synergistic effect. Neurospheres that formed in the absence of FGF2 did not grow as large as fast: the culture time of 7 days was used to ensure an effect of FGF10 or FGF18 could be seen. Although positive control neurospheres proliferated extensively and fused to generate fewer large spheres, comparison of the other conditions reveals a trend for more small neurospheres in FGF10, and larger spheres in FGF18 (figure 5.4B). No co-operative effect of these two growth-factors was seen under these conditions. Despite the lack of an obvious synergy between FGF10 and FGF18, numerous neurospheres suggests FGF10 is a survival factor that maintains neurospherogenic cells, while large neurospheres suggest FGF18 increases proliferation.

22 Fgf ligands have been identified in mammals, and there are only 4 receptors, although isoforms exist. Fgf2, a mitogen, acts through FgfR2c, FgfR3c and FgfR4, the same receptors to which Fgf18 binds (Mason, 2007). Considering that Fgf18 and Fgf2 act through shared receptors, and both show evidence of enhancing proliferation, the neurosphere assay supports Fgf18 as an endogenous mitogen in the adult ventromedial hypothalamus. Previous data from the Placzek lab have shown that *Fgf18* mRNA is expressed exclusively in the alpha2-tanycyte region, in accordance with the site of FGF2-responsive tanycytes. Together, these data suggest that Fgf18 may regulate alpha2-tanycyte proliferation in-vivo.

Neurosphere formation at clonal density is the accepted standard for neurosphere analysis, and while my initial studies were not performed at clonal density, clonality has been confirmed. To provide evidence that clonal hypothalamic neurospheres could be grown from a single cell, eliminating the risk of fusion, dissociated neurosphere cells were sorted into single wells of 96 well plates and cultured for 10 days in EGF/FGF2. In order to identify any difference in clonal neurosphere formation in the presence of FGF10 or FGF18, these factors were added to EGF/FGF2 media (figure 5.4C). Clonal hypothalamic neurospheres were generated in all conditions except media containing both FGF10 and FGF18, suggesting saturating levels of all four growth-factors is not conducive to neurosphere formation and is indeed toxic at the single cell level. In contrast, addition of FGF10 increases the number of clonal neurospheres, as observed at clonal density. Addition of FGF18 appeared to decrease neurospherogenic efficiency, however the infection rate was high in EGF/FGF2 plates and therefore the calculated neurospherogenic efficiency may not accurately represent the true percentage of sphere-forming cells. The size of clonal neurospheres supports the observations from clonal density neurospheres, identifying more small neurospheres in FGF10 media. The addition of FGF18 does not lead to changes in neurosphere number or size compared to the control, adding further support to shared FgfR binding between Fgf2 and Fgf18. Future clonal neurosphere assays will compare the neurospherogenic efficiency of cells in FGF18 or FGF2, and not together. In addition, hydrogel techniques may be of particular use as they can prevent fusion of cells when plated at clonal density (Cordey *et al.*, 2008).

Neurosphere assays have provided evidence that alpha-tanycytes are neurospherogenic and dependent upon Fgf signalling for proliferation in-vitro. Evidence also supports autoregulation of in-vitro proliferation as neurospheres express *Fgf10* and *Fgf18* mRNA. Culture of neurospherogenic cells in the presence of FGF10 increases the number of spheres formed, suggesting FGF10 improves survival of neurospherogenic cells and neurospheres. Fgf18 shares three Fgf receptors with Fgf2, and evidence suggests Fgf18 acts as a mitogen, similarly to Fgf2, in this in-vitro culture system. Additionally, in the absence of definitive markers for fluorescence-activated cell sorting of single subtypes of tanycytes, the formation of clonal neurospheres from single 4° cells adds further support to the self-renewal potential of the alpha2-tanycyte population.

### 5.3.3: Hypothalamic neurospheres are responsive to excitatory neurotransmission

An increasing number of recent studies have implicated neural circuit activity in regulating proliferation and neurogenesis from the SVZ and SGZ of the postnatal and adult rodent. The receptor for the major inhibitory neurotransmitter, $\gamma$-aminobutyric-acid (GABA), is expressed on quiescent radial neural stem cells in the SGZ of the dentate gyrus (Song *et al.*, 2012). Conditional deletion of the GABA receptor in this population results in stem cell proliferation, while GABA signaling regulates quiescence. In contrast, the major excitatory neurotransmitter, glutamate, increases proliferation of neural precursors in the SVZ (Brazel *et al.*, 2005b). As it is important to define the hypothalamic response to neural circuit activity, a physiological response, the neurosphere assay was used as model to assess whether neurospherogenic cells are similarly responsive to NMDA receptor activity (figure 5.5).

The specific pharmacological agonist of the NMDA receptor, NMDA, was added at 10μM and 100μM concentrations to determine the neurospherogenic efficiency compared to control media, containing EGF and FGF2. Results indicate an increase in neurospherogenic efficiency with increasing NMDA concentration: indeed at 100μM concentrations a decrease in neurosphere number combined with increased neurosphere size suggests extensive proliferation and fusion (figure 5.5A). Thus, in concurrence with SVZ and SGZ neurospherogenic cells, NMDA receptor activation increases proliferation of hypothalamic neural stem/progenitor cells in-vitro.

Neurospheres are heterogenous with respect to cellular composition, consisting of stem cells, proliferative progenitor cells and differentiating cells. As it is not clear whether NMDA exerts its proliferative effect on the initial neurospherogenic cell, or a more committed cell type, the neurosphere assay was used to determine its value in distinguishing cellular response. Dissociated cells were plated at clonal density to generate 5° neurospheres in the presence or absence of 10μM NMDA (figure 5.5B). As in the previous experiment, NMDA increased the neurospherogenic efficiency. If NMDA increases proliferation of progenitors, after dissociation of neurospheres a discrepancy in neurospherogenic efficiency would be expected when cultured in control media, compared to control neurospheres. One would hypothesise that plating more progenitors would lead to smaller neurospheres and less total neurospheres as their restricted proliferative program would be diminished in the second passage. Quantifying neurosphere number, and calculating cell number and neurospherogenic efficiency indicates no difference between control spheres and spheres previously subjected to NMDA treatment. This result suggests NMDA exerts its effect at the same level as EGF and FGF2, eliciting an accumulative response. However, it may be appropriate to continue passaging these neurospheres to identify any changes over long-term serial passages.

These results suggest NMDA does not effect solely the progenitor population, although it is possible that NMDA acts on all cell types within the sphere. However, the consideration that further passages are required to force an observable difference highlights the limitations of this in-vitro assay (Ruau *et al.*, 2008; Vukicevic *et al.*, 2010). In actuality, great care must be taken not to misinterpret neurosphere data in the absence of corroborative in-vivo analysis. Despite the caveats and limitations of the neurosphere assay (Pastrana *et al.*, 2011), it remains an excellent tool to investigate self-renewal, proliferation and differentiation at the cellular and molecular level, as well as providing a valuable model for neurological cancer development and drug discovery (Wan *et al.*, 2010; Jensen and Parmar, 2006).

The increase in neurospherogenic efficiency in the presence of NMDA does support a proliferative response of alpha2-tanycytes to glutamatergic signaling, and thus neural circuit activity. This interpretation is in agreement with observations from the SVZ and SGZ of the adult rodent brain, and suggests alpha2-tanycytes are receptive to physiological stimuli that influence excitatory neurotransmission in the hypothalamic nuclei. Further study is required to confirm the responsiveness of alpha2-tanycytes to physiological stimuli in-vivo. Importantly, the neurosphere assay presents an inexpensive and beneficial tool to focus future investigation in the 3$^{rd}$ ventricle. Moreover, the neurosphere assay supports the presence of a self-renewing, multipotent stem cell in the alpha2-tanycyte subtype, and has demonstrated the requirement and autoregulation of Fgf signaling in these cells.

# Chapter 6

## Optimisation of the organotypic slice culture assay as a tool to investigate the hypothalamic niche

## 6.1: Introduction

Culturing neural tissue in a manner that maintains the cytoarchitecture has proven to be a valuable tool, in particular for electrophysiological and pharmacological studies, as individual neurons and networks can be accessed and their response recorded in different conditions. Furthermore, such cultures can be maintained long-term ex-vivo, providing the ability to follow neural differentiation, dendritic branching and synaptogenesis, amongst other significant neurological processes, in their natural surroundings and without the necessity of primary cultures (Kamada *et al.*, 2004). This methodology, termed organotypic slice culture, has been utilised since its introduction in 1947 by Hogue, but has been advanced and optimised by many others (Gahwiler *et al.*, 1997).

Initial studies that maintained viable tissues were performed using the roller-tube technique, in which slices are embedded in a plasma clot on a glass coverslip, and slowly rotated in a tube containing culture media. The rotation of the tube leads to a thinning of the slice over time, eventually resulting in a organised monolayer of cells that have alternating access to oxygen and nutrients, and can therefore be maintained in culture long-term (Gahwiler *et al.*, 1997). In addition, the monolayer is suitable for electrophysiological recordings and (confocal) visualisation of individual neurons.

The established roller-tube method has been followed by two adaptations, the membrane interface and collagen embedding method. The membrane interface method consists of culturing slices on a semiporous membrane that allows diffusion of nutrients and media across the tissue slices, while also freely allowing gas exchange (Stoppini *et al.*, 1991; Gahwiler *et al.*, 1997). Embedding in collagen provides a substrate that allows diffusion of nutrients while maintaining the structural integrity of the tissue; however gas exchange is not facilitated and therefore these cultures are not ideal for long-term cultures of weeks and months (Gahwiler *et al.*, 1997; Placzek and Dale, 1999). These classical slice culture techniques are not mutually exclusive, as they all have complementary advantages and disadvantages that the user must assess, choosing the appropriate method for a particular question.

Organotypic slice cultures are traditionally used to study populations of neurons, however their value as a tool in investigating neural stem cells is increasingly appreciated. Organotypic slice cultures are being used in parallel with in-vivo work, providing a useful complementary system to investigate the response of stem/progenitor cells in their niche. The accessibility to the tissue offers an opportunity to perform microinjections and electroporation of niche cells to deliver mRNA or retroviral vectors in order to lineage trace or change the gene expression of progenitor cells (Taverna *et al.*, 2012). Such studies have been performed in the embryonic CNS, the adult SGZ and the SVZ (Kamada *et al.*, 2004; Dayer *et al.*, 2008; Brunne *et al.*, 2010). In addition, a number of studies have exploited the advantages of the organotypic slice culture system, such as the controlled environment, to address the functional integration of embryonic and neural stem cells into established tissues (Herlenius *et al.*, 2012). Optimisation of such a technique for the study of progenitors in the 3rd ventricle might therefore be invaluable in the progression of our understanding of factors that regulate proliferation, neurogenesis and integration into circuitry.

In this chapter, I assess the merits of the membrane interface and collagen embedding methods of organotypic slice culture. I consider the ability of each to maintain the integrity of hypothalamic niche cells, as a model of the in-vivo environment, and for further studies to determine the proliferative response of progenitors to different conditions.

### 6.2.1: Obtaining slices of the tuberal hypothalamus

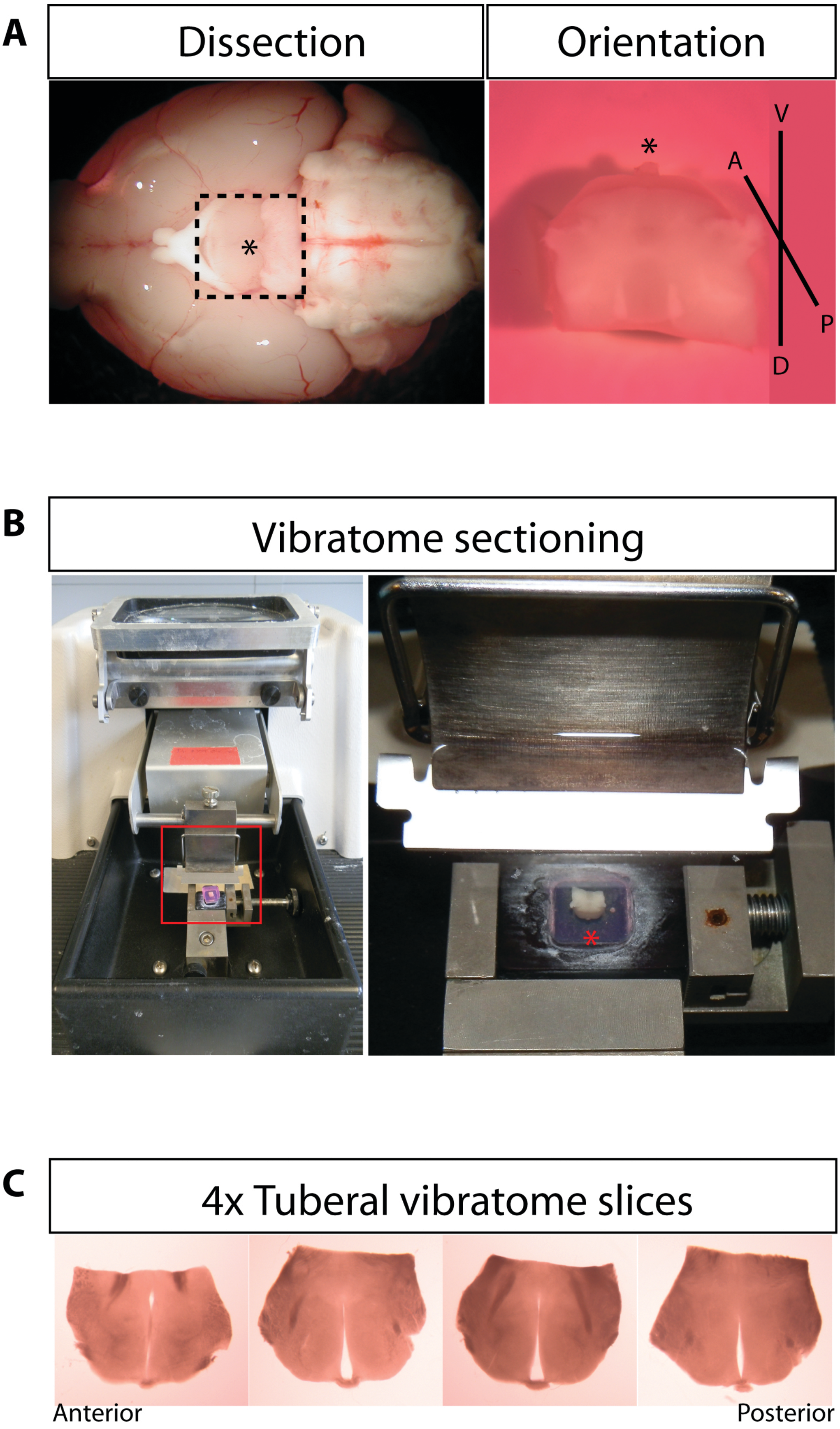
In order to obtain fresh hypothalamic slices, suitable for culture, dissections and processing must be performed efficiently and with sterile equipment to prevent prolonged exposure to infection and an environment that is non-conducive to tissue survival. I therefore aimed to establish a protocol for the routine dissection and culture of slices containing prospective niche cells (i.e. tanycytes) maintained in their environment.

Wherever possible, tissue was kept on ice to decrease cellular metabolism and maintain survival. The hypothalamus was dissected as a cube, providing suitable surfaces for orientating and mounting within agarose (figure 6.1A). A range of agarose concentrations were examined for their ability to support the sectioning: 3% agarose was found to be optimal in supporting the integrity of the tissue during slicing compared to lower percentages. A range of orientations was similarly examined: this revealed that optimal sections were obtained when tissue was orientated so that the median eminence was visible and the ventral side of the hypothalamus was mounted vertically, with the anterior side of the hypothalamus forming the base of the mounted tissue. This method ensured minimal variability between sectioning of different hypothalami and a standard protocol to maximize efficiency.

Set agarose, with the hypothalamus embedded, was removed from its mould and glued onto a chuck, then placed onto the stage of a vibratome, submerged in ice-cold PBS (figure 6.1B). The median eminence faced away from the razor blade to reduce the chance of damage to the ventral 3rd ventricle. A range of slicing conditions were examined to optimise the procedure. The thinnest slices that could be obtained consistently without damage were at a relative thickness of 200μm, at a speed of '6' and amplitude of '7'. These settings generated four tuberal hypothalamic slices with the median eminence intact (figure 6.1C). Hypothalamic slices were collected in ice-cold Leibovitz-15 media to support survival in non-physiological conditions before culture. A change in morphology was observable between each slice; however, four slices were analysed against four slices of equivalent morphology in all experiments, providing comparable samples of the tuberal hypothalamus. This protocol consistently generated high-quality slices of the hypothalamic region in which tanycytes are present, providing starting material for comparing the value of different culture regimes.

**Figure 6.1: Obtainment of tuberal hypothalamic slices.**

A. Wholemount image shows ventral view of mouse brain; the dashed line indicates levels at which a scalpel is used to dissect the hypothalamus as a cube of tissue. The cube of tissue is orientated in 3% (w/v) agarose in HBSS, within a plastic mould, according to the annotated orientation. Asterisk indicates the position of the median eminence (*).
B. The mounted hypothalamus is glued to a chuck and secured onto the vibratome stage with the median eminence facing away from the razor blade (*). The vibratome bath is filled with ice-cold PBS, and hypothalamic slices are cut at a relative thickness of 200μm, a speed setting of '6' and amplitude of '7'. The magnification of the red box is shown in the right-hand panel.
C. Four 200μm tuberal slices of the hypothalamus are obtained from each brain, shown anteriorly to posteriorly. Note the presence of the median eminence and the morphology of the 3rd ventricle as landmarks for orientation and a sign of successful slicing.


A
Dissection
*
Orientation
*
V
A
P
D
B
Vibratome sectioning
*
C
4x Tuberal vibratome slices
Anterior
Posterior

### 6.2.2: The efficacy of membrane culture-inserts in the organotypic slice culture of the tuberal hypothalamus

Membrane culture-inserts are frequently used for organotypic slice culture, particularly of hippocampal tissue, because they are simple and maintain good organotypic organisation of the tissue. As already described, the membrane provides an interface between media and air, allowing media to diffuse across the tissue whilst still permitting gas exchange. Furthermore, the membrane is translucent and has no auto-fluorescence, which are valuable characteristics for imaging (Stoppini *et al.*, 1991). These features encouraged me to assess the suitability of membrane culture-inserts in interrogating the niche within the VZ of 3rd ventricle.

Four tuberal slices from a single hypothalamus were arranged upon a membrane culture-insert (figure 6.2A) and cultured with previously-used neurosphere media (see chapter 5) at 37°C and 5% $CO_2$ for 5 days. Images were captured before culture at 0 hours, and at 24, 72, and 120 hours during culture (figure 6.2B). An apparent flattening of the tissue occured after 24 hours and beyond, including a change in morphology of the tissue surrounding the 3rd ventricle (yellow arrowheads). The change in morphology suggests the membrane does not maintain the tissue integrity that would be observed using in-vivo techniques. Further processing of tissue was therefore performed to further determine the suitability of the membrane in investigating the hypothalamic niche.

Antibodies against phosphorylated Histone-3 (PH3) were used to detect proliferating cells in mitosis (Hans and Dimitrov., 2001). In addition, BrdU was added to culture media and BrdU incorporation was assessed to determine cells in S-phase (Cavanagh *et al.*, 2011). Together, these proliferative markers would enable me to assess the suitability of the culture membrane in examining proliferation in the VZ of the 3rd ventricle (figure 6.3). Immediately-fixed tissue did not show any proliferation, as assessed with anti-PH3 or anti-BrdU (figure 6.3 top panels). In contrast, both S-phase cells and M-phase cells were

## Figure 6.2: Usage of membrane culture-inserts for organotypic slice culture of the tuberal hypothalamus.

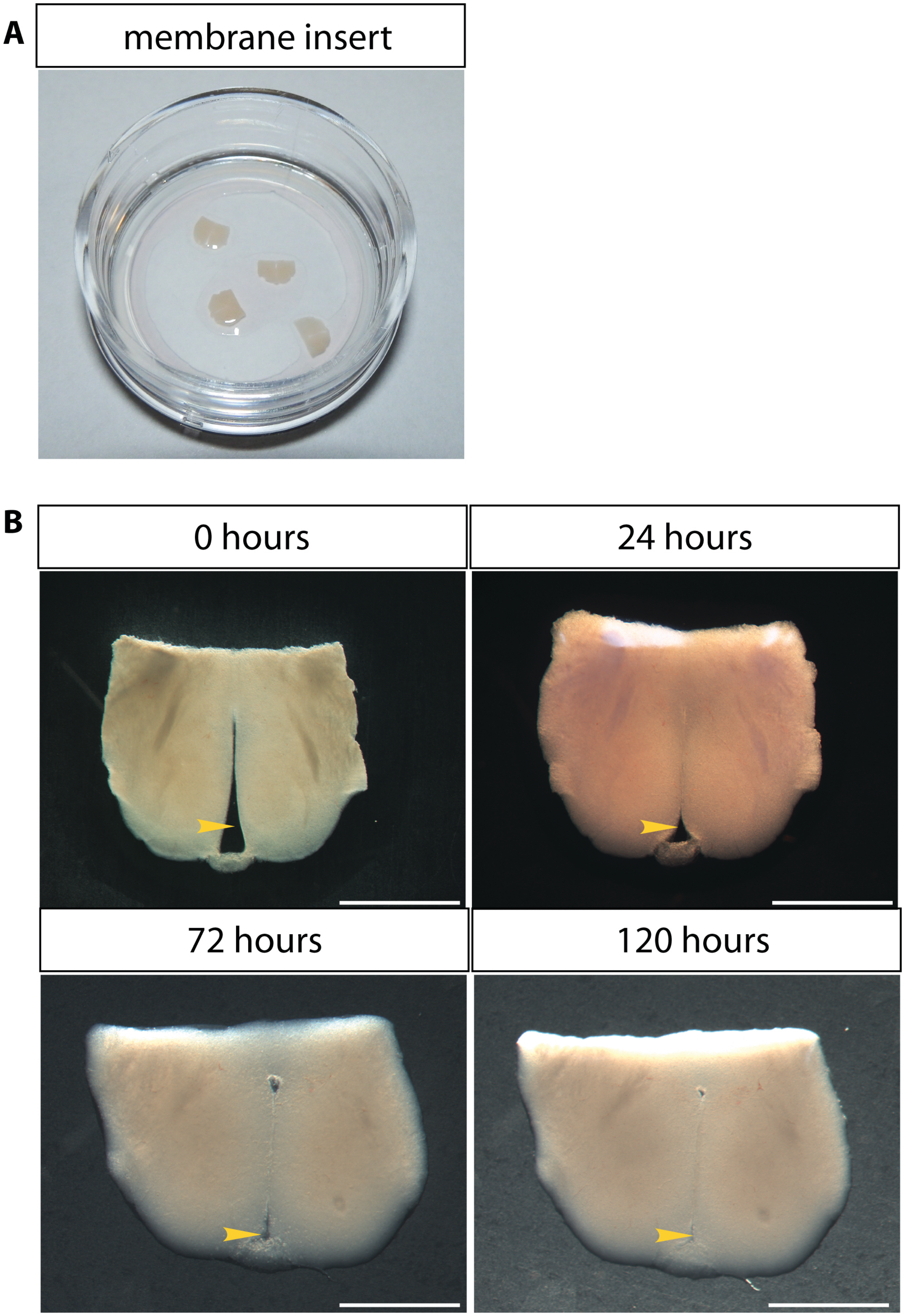
A. A membrane culture-insert is pictured with four tuberal slices arranged upon it. The membrane is inserted into a dish containing culture media for 5 days (120 hours) at 37°C and 5% $CO_2$.

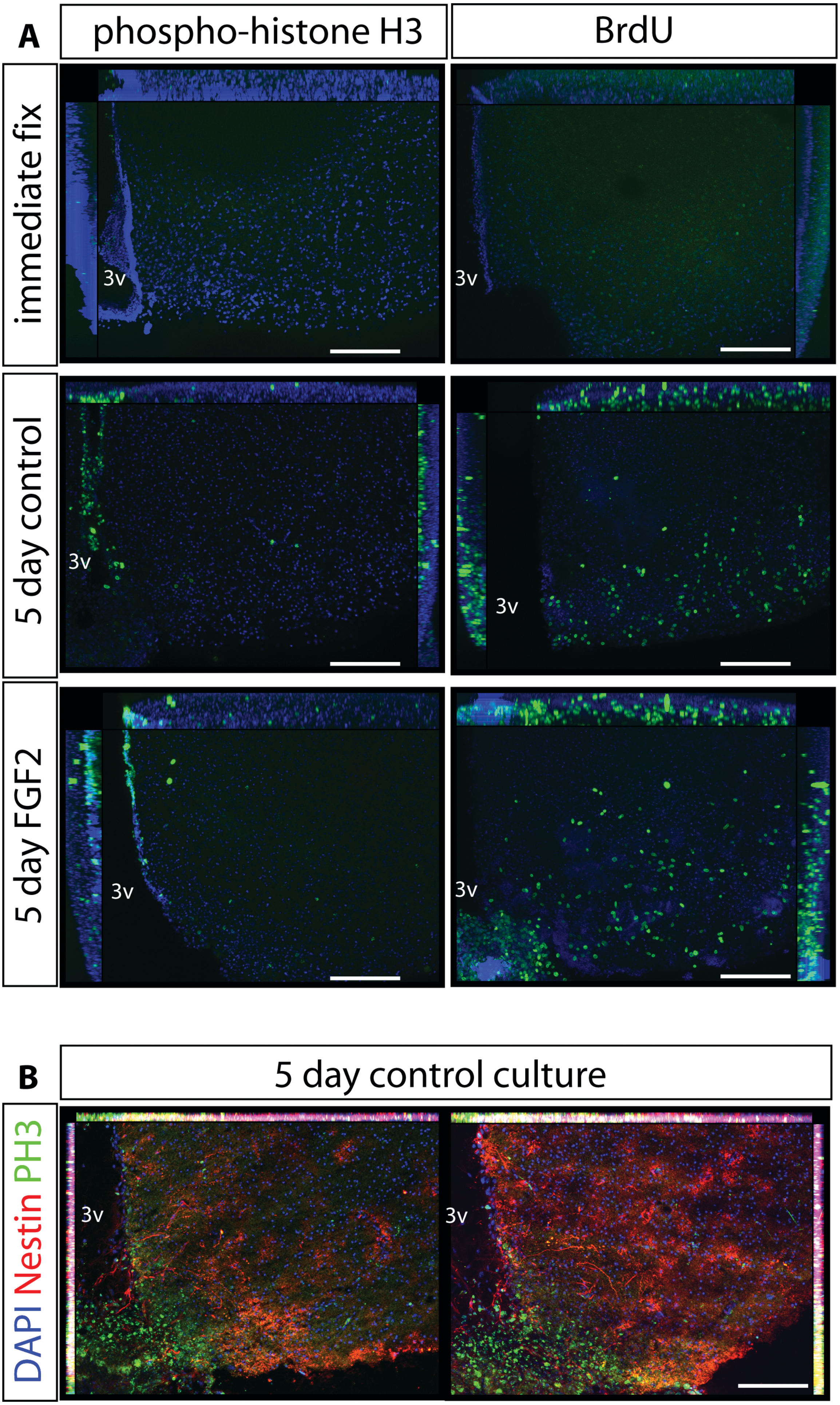
B. The same tuberal slice is imaged at the start of culture (0 hours), then after 24, 72 and 120 hours in culture. A flattening of the tissue and a change in morphology of the 3rd ventricle (yellow arrowheads) is observed. Scale bar represents 1200μm.

## Figure 6.3: Proliferation in organotypic slice cultures on membrane culture-inserts.

A. MIPs are shown for slice cultures. Slices are fixed immediately or cultured for 5 days (120 hours) in culture media containing BrdU, and the addition of FGF2. Slices are immunohistochemically stained for mitotic marker, PH3, and BrdU incorporation (green). DAPI is used as a marker of cell nuclei (blue). PH3 reveals mitotic cells in the VZ, while BrdU incorporation is high parenchymally. The 3rd ventricle is annotated with '3v'. Scale bar represents 100μm.

B. MIPs are shown of slices cultured for 5 days in culture media. Slices are immunohistochemically stained for mitotic marker, PH3 (green), Nestin (red), and DAPI (blue). Nestin reveals poor tanycyte morphology, PH3 indicates high levels of proliferation in median eminence. The 3rd ventricle is annotated with '3v'. Scale bar represents 100μm.


A
membrane insert
B
0 hours
24 hours
72 hours
120 hours


A
phospho-histone H3
BrdU
immediate fix
3v
3v
5 day control
3v
3v
5 day FGF2
3v
3v
B
5 day control culture
DAPI Nestin PH3
3v
3v

detected after culture in control media for 5 days.  PH3-positive cells were detected throughout the VZ of the 3rd ventricle, including the median eminence, suggesting that the membrane culture-insert method leads to aberrant proliferation in this vascularised region. BrdU incorporation did not recapitulate PH3 expression, instead showing sporadic incorporation throughout the parenchyma (figure 6.3A). To determine whether FGF2 (known to stimulate proliferation of alpha-tanycytes in vivo (chapter 4)) altered the pattern of proliferation, FGF2 was added to the medium of sister-slices. The presence of FGF2 appeared to alter the pattern of PH3-expression, enhancing PH3-positive cells in the alpha-tanycyte region, and suppressing PH3-positive cells in median eminence regions. However, care must be taken in interpreting these results, as the median eminence became damaged in the subsequent processing of tissue adhered to membranes. The presence of FGF2 did not, however, alter the pattern of BrdU incorporation. Together, these results do not support the membrane-culture method as a useful tool to examine the hypothalamic niche, as proliferation patterns observed in-vivo are not recapitulated ex-vivo.

To examine this further, tanycytic expression of the intermediate filament protein, Nestin, was next used to assess whether the morphology of the tissue surrounding the 3rd ventricle was maintained relative to in-vivo conditions (figure 6.3B). In many cases (although note there was much inconsistency: data not shown), tissue appeared disorganised, and only sparse tanycyte processes were detected.

Together, the change in tissue morphology in the initial 24-hours, and throughout the rest of the culture period, and the failure to recapitulate the in vivo patterns of proliferation, either in control conditions or after FGF2-treatement, suggest the membrane culture-insert does not support the tissue surrounding the 3rd ventricle, and thus does not maintain the niche appropriately relative to the in-vivo environment.

### 6.2.3: Collagen as an appropriate substrate for the organotypic slice culture of the tuberal hypothalamus

The unsuitability of membrane culture-inserts in investigating the hypothalamic niche within the VZ of the 3rd ventricle led me to search for an alternative method of culture, that would protect against changes in tissue morphology and integrity. Collagen gels are traditionally used for the culture of small pieces of dissected embryonic tissue in-vitro, termed explants (Placzek and Dale, 1999). To my knowledge, collagen gels have not been used to research adult stem cell niches as organotypic slice cultures**.** Collagen gels provide a three-dimensional environment that offers structural support to the tissue without suffocation, allowing diffusion of nutrients and growth factors. These characteristics are suitable for the purposes of investigating the neural stem/progenitor cell niche within the VZ of the 3rd ventricle in a short-term, therefore I assessed the integrity and proliferative response of tuberal hypothalamic slices cultured in collagen.

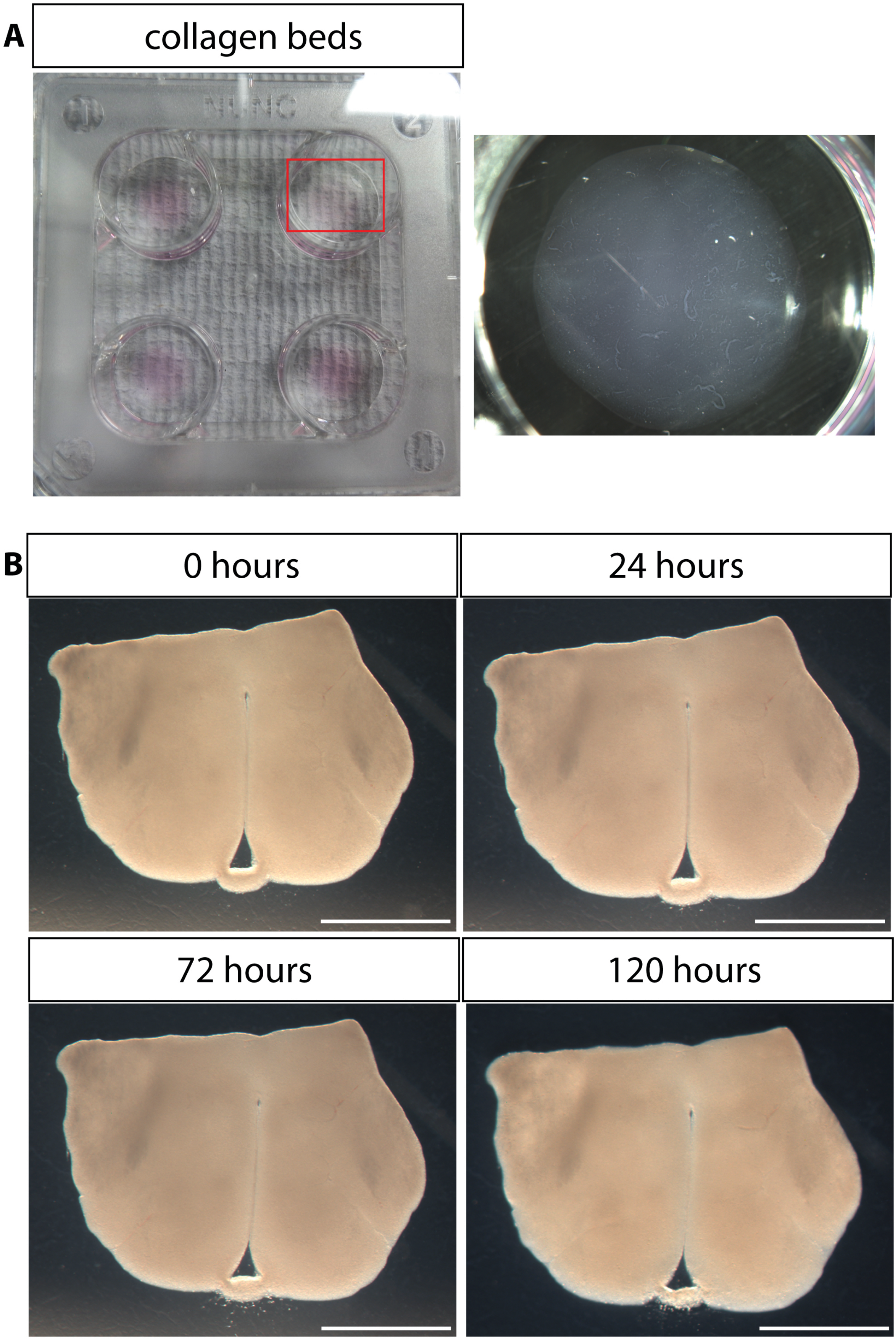
Tissue was embedded in 3-dimensional (3d) collagen gel, in 4-well plates, using standard methods (see Materials and Methods and figure 6.4A). After the collagen had set, equilibrated culture media was added to each well and the slices were cultured for 48 hours at 37°C and 5% $CO_2$. Images were captured before culture, and then at 24, 72 and 120 hours (figure 6.4B). In contrast to the slices cultured on membranes, no change in the gross morphology of the 3rd ventricle could be detected, suggesting that the 3d collagen may maintain tissue integrity in a manner comparable to the in-vivo environment.

To examine this in further details, I analysed PH3 expression after 48h in culture, directly comparing slices cultured in collagen with equivalent slices cultured on a membrane (figure 6.5A). In addition, I compared two culture media: the neurosphere media (NS) used in chapter 5, and used in the membrane cultures described above, and neurosphere media customised to lack insulin and IGF (hereafter referred to as slice culture media (SC)). Culturing slices on membranes in SC media appeared to improve tissue integrity: Nestin-

## Figure 6.4: Usage of collagen beds for organotypic slice culture of the tuberal hypothalamus.

A. Collagen beds are prepared in 4 well dishes, the boxed region is magnified in right-hand panel. After slices are placed on to the collagen bed, further collagen is pipetted on top to envelop the slices. After the collagen has set, culture media is added and the slices are cultured for 5 days (120 hours) at 37°C and 5% $CO_2$.

B. The same tuberal slice is imaged at the start of culture (0 hours), then after 24, 72 and 120 hours in culture. The morphology is maintained during the culture period. Scale bar represents 1200μm.


A
collagen beds
NUNC
B
0 hours
24 hours
72 hours
120 hours

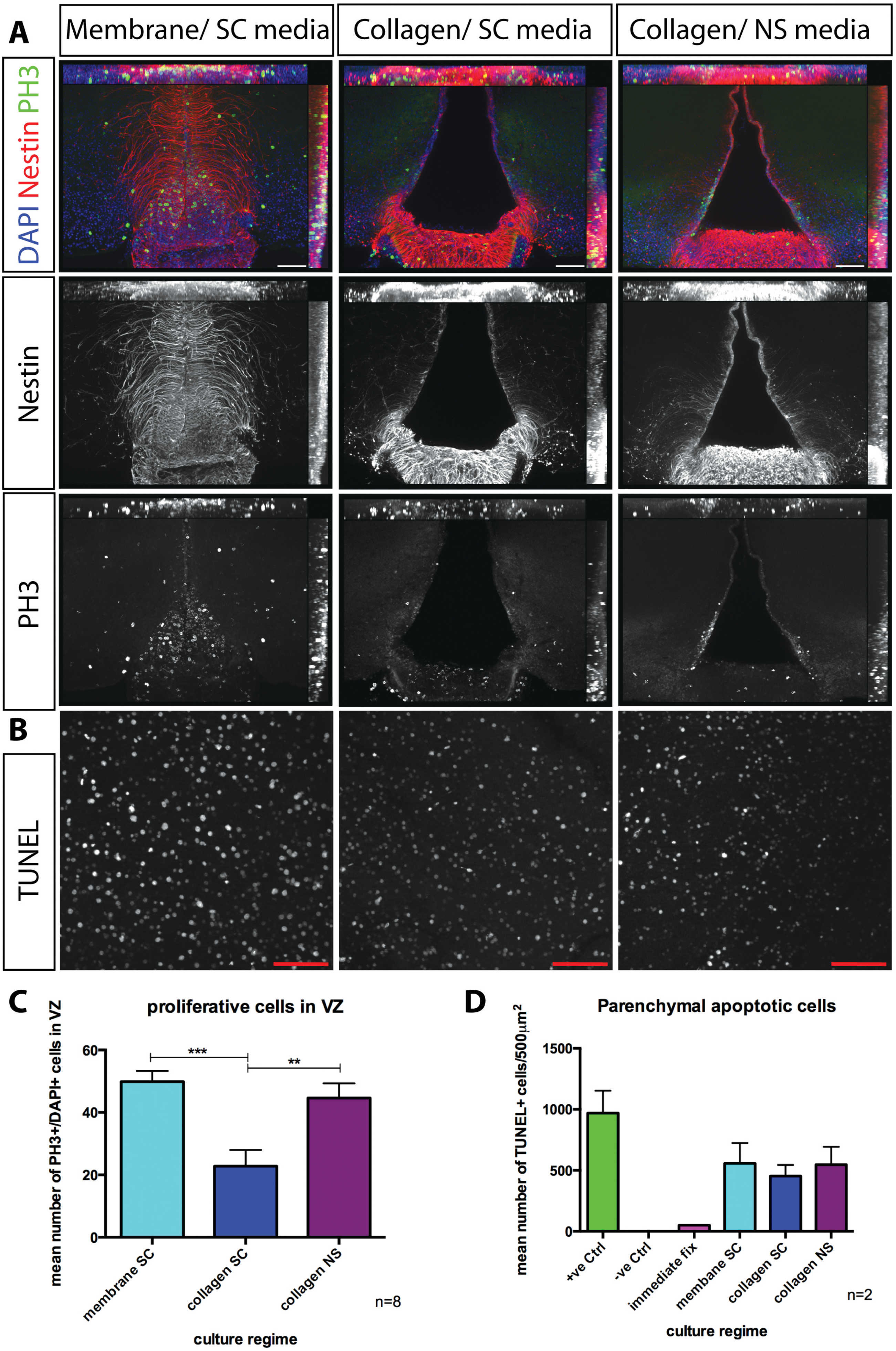
positive tanycytes were maintained (compare figures 6.3 and 6.5). However, the disrupted overall morphology of the slice (left hand panels in figure 6.5) meant that it was impossible to determine tanycyte subtype. Furthermore, although PH3-positive cells were detected, the flattened nature of the slices meant that it was difficult to define PH3-positive cells as ventricular, subventricular or parenchymal. In contrast, slices cultured in collagen maintained their overall morphology, and maintained the same profile of Nestin-positive tanycytes as was detected in vivo (compare figures 4.2A and 6.5 right hand panels). Moreover, it was possible to assign PH3-positive cells to the VZ/SVZ or parenchyma, and to specific tanycyte-subtypes. Comparison of the two different media revealed that media devoid of IGF/insulin (SC) maintained tanycytes and appeared to reduce the number of PH3-positive cells, compared to NS media. This suggests that culture in collagen/ SC media most faithfully recapitulates the in-vivo hypothalamic environment, where proliferation rates are low. Indeed, when quantified, culturing slices in collagen/SC resulted in a significant decrease in proliferation compared with membrane/SC and collagen/NS regimes, with a mean of 22.3 (±5.2) PH3-positive VZ cells per hemisphere (figure 6.5C).

Culturing in collagen might result in increased apoptosis (programmed cell death), due to decreased gas exchange relative to the membrane interface culture method (Gahwiler *et al.*, 1997). To determine whether different culture regimes stimulate apoptosis, I performed terminal deoxynucleotidyl transferase dUTP end-labelling (TUNEL) stains on slices cultured in the different regimes to identify apoptotic cells (figure 6.5B). Apoptotic cells were observed in all regimes. The number of apoptotic cells was quantified in a randomly selected 500$\mu m^2$ region of hypothalamic parenchyma (figure 6.5D). As a positive control, DNase was used to create DNA ends available for dUTP end-labelling, while no transferase enzyme was added to end-labelling solution in negative controls. No significant difference was observed in the amount of apoptosis between culture regimes (figure 6.5D). In addition the low level of apoptosis in tissues fixed immediately after slicing indicates that vibratome sectioning does not upregulate controlled cell death, and confirms it as a reliable method for slicing fresh tissue. The TUNEL assay suggests ex-vivo culture of hypothalamic slices upregulates

## Figure 6.5: Comparative analysis of culture substrates and media.

Two culture substrates, membrane and collagen; and two culture media, neurosphere (NS) and slice culture (SC); are compared according to the morphology of tissue, amount of proliferation and number of apoptotic cells. Slices are cultured for 48 hours at 37°C and 5% $CO_2$.

A. MIPs of the tuberal hypothalamus are shown, immunohistochemically stained for DAPI (blue), Nestin (red) and PH3 (green). Nestin shows tanycytes are present after each culture regime, but a flattened morphology is apparent with membrane culture inserts. PH3-positive cells are abundant in membrane/SC and collagen/NS cultures. Collagen/SC cultures maintain morphology and fewer PH3-positive cells are apparent. Scale bar represents 100μm.
B. MIPs of 500$\mu m^2$ of the tuberal parenchyma are shown, labeled for apoptotic cells using TUNEL staining. Apoptotic cells are observed in parenchymal tissue of each culture regime. Scale bar represents 100μm.
C. The mean number of PH3+/DAPI+ cells in the hypothalamic VZ of 8 hemispheres is quantified for each culture regime. A significant decrease in proliferative cells is calculated from both membrane/SC and collagen/NS cultures to collagen/SC cultures ($p<0.05=*$). Error bars represent SEM. ($n=8$).
D. The mean number of TUNEL-positive cells in 500$\mu m^2$ of parenchymal tissue is quantified for each culture regime. Positive controls are treated with DNase, negative controls are not treated with TdT enzyme. Immediately fixed slices are analysed to detect apoptosis in response to vibratome slicing. Vibratome slicing does not result in an upregulation of apoptosis in immediately fixed slices. No significant difference in the number of apoptotic cells is calculated between the culture regimes. Error bars represent SEM. ($n=2$).


A
Membrane/ SC media
Collagen/ SC media
Collagen/ NS media
DAPI Nestin PH3
Nestin
PH3
B
TUNEL
C
proliferative cells in VZ
mean number of PH3+/DAPI+ cells in VZ
60
40
20
0
***
**
membrane SC
collagen SC
collagen NS
n=8
culture regime
D
Parchymal apoptotic cells
mean number of TUNEL+ cells/500µm²
1500
1000
500
0
+ve Ctrl
-ve Ctrl
immediate fix
membane SC
collagen SC
collagen NS
n=2
culture regime

apoptosis, but that no difference is observed between membranes and collagen, or NS media and SC media.

These data support collagen gels as a suitable substrate for the three-dimensional culture of hypothalamic slices, maintaining their integrity. Furthermore, they highlight SC media as maintaining a reduced level of proliferation compared to NS media. In light of these results, future experiments all used the collagen/SC regime as standard, including a 24 hour equilibration/recovery period, and a 24 hour experimental culture period.

### 6.2.4: Tanycyte response to FGF2-infusion in-vivo is recapitulated by organotypic slice culture assay

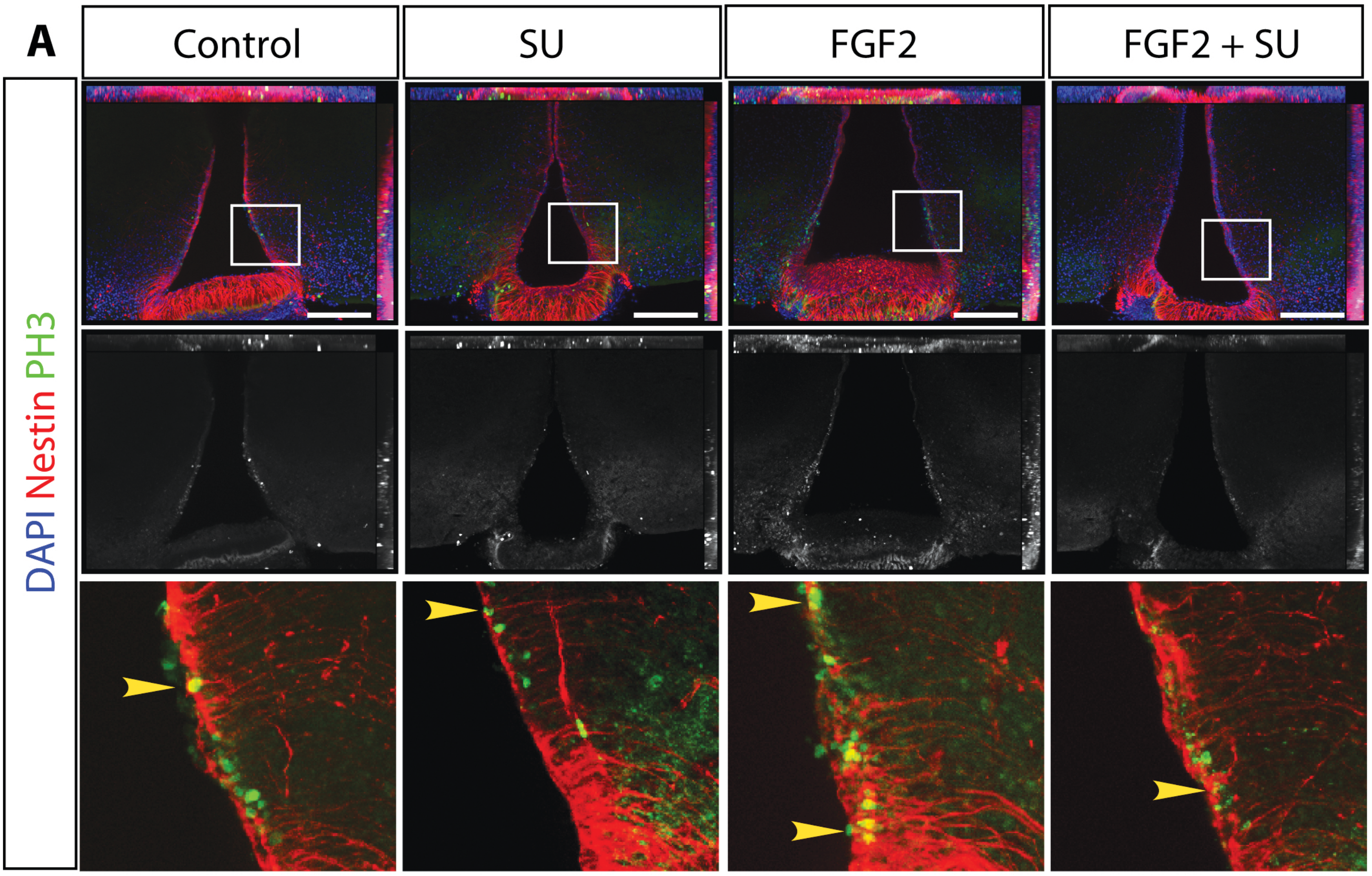
To test the efficacy of the organotypic slice culture regime, hypothalamic slices were treated with FGF2 to determine whether the in-vivo infusion result (chapter 4) was recapitulated. In addition, the ex-vivo regime provided an opportunity to inhibit Fgf receptors without systemic consequences.

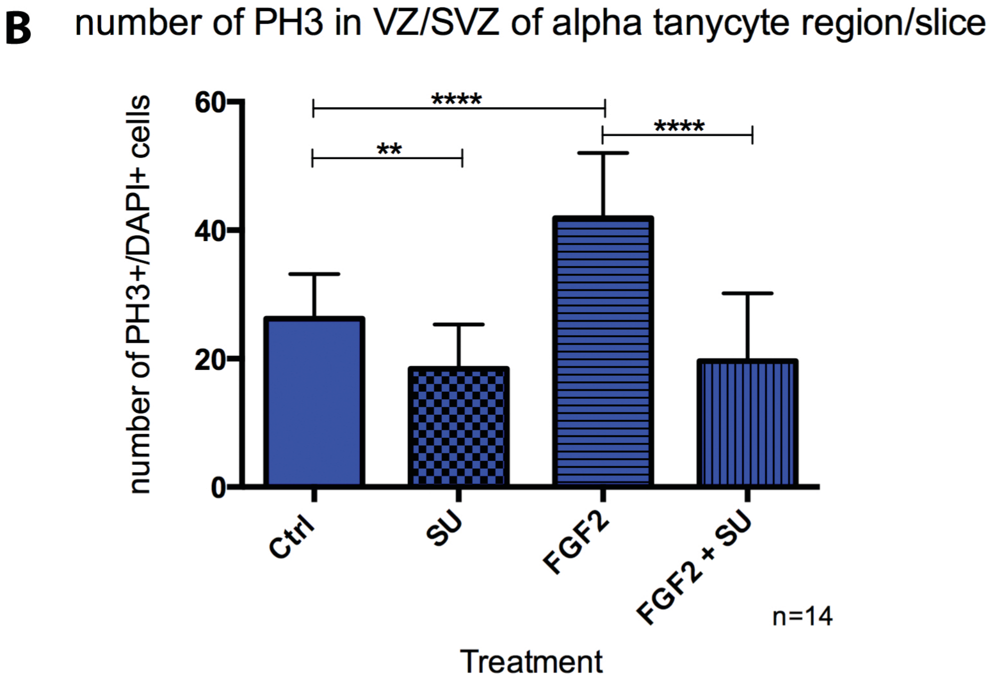
After the 24-hour culture equilibration/recovery period, tuberal hypothalamic slices were cultured in SC media alone, media containing FGF2, the FgfR inhibitor, SU5402, or both, for a further 24 hours. Slices were fixed and immunolabelled for PH3 and Nestin (figure 6.6A). PH3-positive cells were observed in all conditions; however FGF2 appeared to stimulate the number of PH3-positive cells in the VZ, whereas proliferation appeared decreased in media containing the Fgf inhibitor, SU5402.  Significantly, VZ proliferative cells appear to be restricted to the alpha-tanycyte subtypes, both in controls and after culture with FGF2. Double-label analyses revealed that Nestin-positive alpha-tanycytes appeared to co-express PH3 in all conditions, with an apparent upregulation in FGF2 media, supporting the idea that alpha-tanycytes proliferate in response to Fgf signalling. These results are consistent with our in-vivo infusion data that show that alpha-tanycytes are responsive to FGF2.

## Figure 6.6: Organotypic slice culture assay recapitulates in-vivo FGF2 infusion assay.

Control slice culture media, media containing FGF2 (60ng/ml) or Fgf receptor inhibitor, SU5402 (20μM), or media containing both FGF2 and SU5402 is added to hypothalamic slices cultured in collagen following a 24hour recovery period. The slices are cultured in experimental media for 24 hours. Slices are cultured at 37°C and 5% $CO_2$.

A. MIPs are shown for tuberal hypothalamic slices immunohistochemically stained for Nestin (red), PH3 (green) and DAPI (blue). Black/white panels are immunostained for PH3. Tanycytes are maintained in each culture regime. Proliferative cells are observed in the VZ of all culture regimes, with differences in numbers. Boxed regions are magnified in bottom panels. Yellow arrowheads indicate examples of PH3+/Nestin+ alpha-tanycytes. Scale bar represents 100μm.
B. The mean number of PH3+/DAPI+ cells in the hypothalamic VZ of 14 hemispheres is quantified for each culture treatment. A significant increase in PH3+ VZ cells is calculated in FGF2 treated slices compared to control and media containing FGF2 and SU5402. A significant decrease in PH3+ VZ cells is calculated in slices treated with SU5402 alone compared to the control ($p<0.05=*$). ($n=14$).




number of PH3 in VZ/SVZ of alpha tanycyte region/slice

Quantitative analyses further support this conclusion. Quantification of PH3+ cells in the VZ revealed that FGF2 significantly increases proliferative cells, compared to control media and media containing Fgf inhibitor. Conversely, addition of SU5402 reduces the number of proliferative cells below the control, suggesting that in the absence of exogenous FGF2, local Fgf signalling stimulates proliferation. The presence of some proliferative cells in SU5402-treated slices may suggest that the inhibitor is not 100% efficient, or may suggest that Fgf-independent factors also regulate proliferation in VZ of the 3$^{rd}$ ventricle.

Taken together, these data support organotypic slice culture in collagen gels as a reliable method for investigating the proliferative response of tanycytes ex-vivo, and add further insight to our in-vivo studies, revealing that Fgf signalling is required for proliferation of alpha-tanycytes. This supports the use of organotypic slice culture as a robust system to focus investigations prior to testing in-vivo.

## 6.3: Discussion

My previous studies identified an adult neural stem/progenitor population in the alpha-tanycyte subtype (chapter 4). The in-vitro neurosphere assay confirmed and extended these results, identifying alpha2-tanycytes as harbouring neurospherogenic potential that is maintained long-term (chapter 5). The limitations of the neurosphere assay encountered in analysing the response of progenitor cells to physiological stimulation led me to identify an alternate culture technique, which can be used to complement my previous studies, to assess the response of progenitors maintained in their niche as a model for the in-vivo environment.

The viability of tuberal hypothalamic slices on membrane culture-inserts and embedded in collagen, maintained short-term for a maximum of 5 days, was assessed by consideration of the gross morphology of the 3$^{rd}$ ventricle, the presence of tanycytes, the number of proliferative cells and the amount of apoptosis. Here, I discuss the suitability of the membrane interface and the collagen embedded organotypic slice culture assays in characterising the proliferative response of tanycytes to factors for future studies.

### 6.3.1: Gross morphology of the 3$^{rd}$ ventricle

Tuberal hypothalamic slices were arranged on a membrane culture-insert or in collagen beds and cultured for 5 days (120 hours) to determine the extent of morphological change around the 3$^{rd}$ ventricle over this period. This is of significance as changes in structural integrity, compared to the in-vivo environment, may alter the physical relationship between niche cells. In this case a model, however informative, may provide an inaccurate representation of in-vivo conditions. Considering that my intention was to optimise an assay that recapitulates the in-vivo progenitor response, I felt it was important that the slice cultures maintained a similar gross morphology to the morphology found in vivo.

Vibratome-slicing was found to result in hypothalamic slices that show the same tissue organisation found in-vivo (figure 6.1C). However, when placed on a membrane culture-insert, a flattening of the tissue and subsequent change in morphology of tissue around the 3$^{rd}$ ventricle was observed as early as 24 hours in culture (figure 6.2B). Over time, the flattening of the tissue resulted in a spreading and increase in size of the hypothalamic slice. After 120 hours in culture the 3$^{rd}$ ventricle appeared to be closed, and is thus not an accurate representation of the morphology observed in-vivo. In contrast, embedding slices in collagen maintained the same morphology over 120 hours, without spreading or closure of the 3$^{rd}$ ventricle (figure 6.4B). Collagen therefore provides a structural support for hypothalamic tissue, which ensures the integrity of the niche cells in the 3$^{rd}$ ventricle.

As studies described in this thesis identify and characterise alpha-tanycytes as component neural stem/progenitor cells of a hypothalamic niche, slice culture conditions that maintain their integrity are of particular value in confirming previous observations and probing the identity, relationship and regulation of niche cells. The maintained three-dimensional cytoarchitecture in collagen, compared to a semi-three-dimensional on membrane culture-inserts, provides initial confirmation of the suitability of the collagen technique for the purposes of this study.

## 6.3.2: Maintenance of tanycytes

As tanycytes appear to be vital components of an adult hypothalamic neural stem/progenitor niche, culture conditions that do not maintain the survival and structure of tanycytes would not be conducive to studies that aim to investigate them. Expression of the intermediate filament protein, Nestin, expressed by neural progenitors and tanycytes, was used here to identify the presence of tanycytes and their basal processes as a method to confirm the suitability of culture conditions.

After 5 days of culture on a membrane culture-insert in NS medium, Nestin-positive tanycytes were sparse and appeared disorganised compared to the observed expression pattern in-vivo (figure 6.3B). This suggests that the membrane interface does not support tanycyte integrity long-term. The membrane interface method was therefore adapted (using SC medium) and compared to the collagen method after 48 hours in culture (figure 6.5A). After 48 hours in SC medium, Nestin-positive tanycytes were maintained on membrane culture-inserts; however the change in morphology around the 3rd ventricle made it difficult to identify tanycyte subtypes by location and projection. In the absence of tanycyte subtype-specific markers, the membrane interface is not appropriate for distinguishing tanycyte responses. However, this assay may prove valuable for future studies that utilise transgenic animals, as specific cell types will be able to be visualised and the flattened morphology makes the tissue more amenable to microscopy.

Maintained nestin-positive tanycytes were also observed after 48 hours when hypothalamic slices were cultured in collagen (figure 6.5A). In addition, the maintained structure of the $3^{rd}$ ventricle made it possible to identify tanycyte subtypes according to location and projection. These features of the collagen technique make it suitable for investigating the $3^{rd}$ ventricle niche with respect to distinguishing responses between tanycyte subtypes. A disadvantage to the collagen system, however, is the fact that the maintained VZ organisation keeps tissue thick and can therefore only be visualised by taking image sections throughout the tissue. While the presence of the ventricle makes it possible to see cells throughout the VZ, it is difficult to achieve a level of resolution required for long-term lineage tracing studies.

### 6.3.3: Proliferation in the VZ of the $3^{rd}$ ventricle

Proliferation results in the generation of two daughter cells from the cell of origin, which are either identical as a result of symmetric division, or non-identical as a result of asymmetric division. In asymmetric division, a cell may retain progenitor characteristics while a process of lineage restriction and differentiation is activated in the other. As an increase in proliferation of neural stem/progenitor cells is often associated with an increase in neurogenesis or gliogenesis (chapter 1), being able to identify changes in proliferative response in short-term cultures is a useful tool in identifying factors that may be neurogenic in the long-term. It is therefore important to ascertain a reliable marker of proliferation, and to characterise control culture conditions that maintain a relatively low level of proliferation.

Incorporation of BrdU is an established method to identify cells that have undergone DNA synthesis during proliferation (Cavanagh *et al.*, 2011). Expression of phosphorylated Histone3 (PH3) is also an accepted marker of cells undergoing mitosis (Van Hooser *et al.*, 1998; Hans and Dimitrov, 2001). Antibodies against BrdU and PH3 were used here to determine the usefulness of each marker and the amount of proliferation in control conditions. Initially, PH3 and BrdU were assessed in 5 day cultures on membrane culture-inserts to determine the suitability of the two markers (figure 6.3A). Immediately-fixed slices did not show immunoreactivity to PH3 or BrdU, confirming that proliferation is low in uncultured hypothalamic tissue and that BrdU does not react non-specifically. After 5-days in culture, PH3 positive cells could be seen in the VZ of the 3rd ventricle, but no BrdU was incorporated in the VZ. As my previous studies have shown that FGF2-infusion stimulates proliferation in the alpha2-tanycyte region, FGF2 was added to culture media to determine whether BrdU incorporation in this region would follow. While PH3-positive cells were potentially more restricted to alpha2-tanycytes after FGF2-treatment, BrdU did not appear to be incorporated in the alpha-tanycyte region. However, the VZ appeared damaged. Together, these results suggest: (1) that the membrane culture-insert can result in damage to the VZ during processing; and (2) that BrdU is not as useful as PH3 in identifying proliferative cells in the VZ. Considering that BrdU can induce senescence of neural progenitor/stem cells (Ross *et al.*, 2008), the exposure of slice cultures to BrdU for the study of proliferation may not be appropriate. Furthermore, BrdU is incorporated during DNA repair and apoptosis (Taupin, 2007), shown to be upregulated in ex-vivo conditions (figure 6.5B,C). Therefore PH3 was used in subsequent experiments to identify proliferative cells specifically.

VZ cells were then analysed for PH3 expression in membrane inserts and collagen; in addition, neurosphere media and a customised neurosphere media, termed slice culture media, were compared (figure 6.5A). Neurosphere medium was used as I had previously found that it maintains progenitors when cells are dissociated. However, previous studies have shown that insulin-like growth factor-1 (IGF-1) stimulates proliferation in the alpha-tanycyte region (Perez-Martin *et al.*, 2010), therefore I wished to analyse a customised neurosphere medium (‘slice culture’ medium), made without insulin and IGF. In membrane culture-inserts cultured with slice culture media, high numbers of PH3-positive cells were observed in the ventral VZ, despite the absence of insulin/IGF. Similarly, hypothalamic slices cultured in collagen with neurosphere media generated high numbers of VZ PH3-positive cells in the alpha-tanycyte region (figure 6.5A). By comparison, significantly fewer VZ PH3-positive cells were detected in the collagen cultures exposed to SC medium (figure 6.5C).

An important technical note is the inclusion of a 24-hour culture period before experimentation. Progression of progenitors through the cell cycle is considered to last 24 hours (Ponti *et al.*, 2013), therefore to limit the possibility of a tissue processing-induced artefact on proliferative numbers, a 24-hour culture period was used, on the basis that any proliferation caused by processing of tissue would be excluded from the analysis. This initial 24-hour period therefore provided time for the tissue to equilibrate to culture conditions, and recover from the tissue-stress of dissection and slicing.

Together these results support PH3 as a suitable marker for identifying proliferative cells and indicate that the customised slice culture media, devoid of IGF and insulin, results in low proliferation and therefore best recapitulates in-vivo conditions.

## 6.3.4: Stimulation of apoptosis by culture conditions

TUNEL analyses reveal that, compared to immediate-fixed tissue, culture increases the number of apoptotic cells. However, no significant difference in the number of apoptotic cells could be calculated between culture regimes, although it should be noted that, although a large area was analysed, the number of repeats is low. It is important to note that quantifying the number of apoptotic cells does not give a measure of uncontrolled cell death, necrosis, which may occur as a result of tissue suffocation. However, as hypothalamic slices are thin (200μm) and cultured short-term, the level of necrosis is unlikely to affect results. Indeed, the presence of the 3rd ventricle facilitates nutrient diffusion through the tissue, and no discolouration of media due to pH changes, associated with cytotoxicity, was observed (personal observation).

In conclusion, hypothalamic slices cultured in collagen short-term did not exhibit an increase in apoptotic cells compared with the membrane interface culture method, a method established to provide gas exchange. This suggests the non-facilitation of gas exchange in collagen is not a significant problem in 200μm thick hypothalamic slices. Furthermore, the removal of insulin and IGF did not increase the levels of apoptosis, suggesting that SC media is able to maintain viability of tissue at a comparable level to neurosphere media. Such assays should be re-tested in thicker tissues or longer cultures to avoid assumptions that programmed cell death does not increase.

### 6.3.3: Value of the organotypic slice culture assay as tool to investigate the adult 3rd ventricular niche

The data above support the collagen/SC regime as a suitable condition to culture tuberal hypothalamic slices for the short-term analysis of progenitor proliferation, maintaining structural integrity of the 3rd ventricle and cells within the VZ. In order to confirm the suitability of this regime in recapitulating the in-vivo conditions, FGF2 was added to SC media in order to determine whether, as with in-vivo FGF2 infusion (chapter 4), alpha2-tanycytes specifically respond by undergoing proliferation (figure 6.6A). In addition, the Fgf receptor inhibitor, SU5402, was added, to extend our previous data by determining whether proliferation was inhibited when Fgf signalling is inhibited.

Tanycytes and their processes were preserved in all conditions, including SU5402, indicating that Fgf signalling is not required for their maintenance. Similarly, PH3-positive Nestin-positive cells were observed in the alpha2-tanycyte region in all conditions (yellow arrowheads) (figure 6.6B). However quantification of the number of PH3-positive cells in the VZ of the 3rd ventricle revealed a significant increase in proliferative cells in response to FGF2 above the control (figure 6.6). These results accurately recapitulate the data from the FGF2 infusion, supporting the organotypic slice culture method as a valuable model for in-vivo conditions. In addition, SU5402 was found to significantly reduce the number of PH3-positive cells in FGF2 treated slices, providing further confirmation that alpha2-tanycyte proliferation is mediated by Fgf signalling. Furthermore, the significant decrease in the number of proliferative cells between the control and SU5402 alone suggests that endogenous Fgf signalling stimulates proliferation in control media. This observation is supportive of previous data that identify Fgf10 and Fgf18 as endogenous factors that can regulate neurospherogenic efficiency (chapter 5). Interestingly, PH3-positive cells were observed in media containing SU5402 alone, possibly suggesting a program of Fgf-independent proliferation. However the method of visualisation does make it difficult to distinguish between PH3-positive cells in VZ compared to the SVZ, therefore it is possible that VZ proliferation is Fgf dependent whilst SVZ proliferation is Fgf independent, with the quantified basal level of proliferation representing the SVZ.

These data confirm and extend the FGF2-infusion data, showing that FGF2 stimulates proliferation in alpha2-tanycytes. Furthermore, inhibition of Fgf signalling supports endogenous Fgfs as stimulators of proliferation, in the absence of exogenous FGF2. In addition, the fact that SU5402 reduces the level of proliferation in the presence of FGF2 confirms that it is indeed the Fgf signalling that stimulates the proliferation in alpha-tanycytes. Such analyses are difficult to perform in-vivo as the injection or infusion of the Fgf inhibitor can have systemic effects: blocking signalling of Fgf receptors as well as blocking vascular endothelial growth factor receptors (VEGFR) and platelet derived growth factor receptors (PDGFR). Such off-target effects are particularly significant in the cardiovascular system and it is, understandably, not ideal to treat mice with SU5402, from an animal welfare perspective.

The organotypic slice culture regime optimised here presents an invaluable tool in assessing the 3rd ventricular niche, requiring only Schedule 1 Home Office Licensing. In contrast, while the niche is retained in its environment, the slice is removed from a biological context and thus subtle or significant systemic effects that may normally occur as a result of an altered regulation of progenitors would remain unidentified. However for the purposes of investigating the cellular response of niche cells to different growth or physiological factors, the organotypic slice culture assay is a highly efficient and robust technique that can be used to focus future in-vivo studies. This is particularly significant in the Home Office's program to reduce, replace and refine. While the same model organism is used, moderate and severe procedures only need be performed after sufficient evidence to support their requirement, obtained ex-vivo. Unfortunately, as four non-comparable tuberal slices are collected from each hypothalamus, it is not yet possible to reduce the volume of mice required and still provide statistically sufficient sample numbers.

In conclusion, while the neurosphere assay is a powerful in-vitro tool to investigate progenitors in the absence of their natural niche environment, the organotypic slice culture assay provides an invaluable tool to extend in-vitro analysis to a system in which the cytoarchitecture of the niche is preserved. Furthermore, it is an inexpensive, rapid and relatively less severe method for focusing further in-vivo investigations. I show here that collagen embedding is the most suitable regime for the short-term analysis of hypothalamic VZ cells, with ventricular morphology and tanycytes being maintained in a manner that recapitulates the in-vivo environment. The neurosphere media devoid of insulin and IGF (SC media) is also shown to be the most appropriate media to maintain tissue viability, tanycyte processes and low levels of proliferation. In addition I provide evidence of the suitability of this technique in recapitulating in-vivo conditions, as the effect of FGF2 on alpha2-tanycytes is recapitulated, while the advantages of the slice culture assay also extends previous analysis. These features validate the benefits of using this organotypic slice culture assay to investigate hypothalamic stem/progenitor cells.

# Chapter 7

## Slice culture assays reveal a proliferative response of the 3$^{rd}$ ventricle niche to physiological stimuli

## 7.1: Introduction

My optimised slice culture assay (chapter 6) confirmed and progressed my understanding of the regulation of alpha-tanycytes as adult hypothalamic neural stem/progenitor cells. Following the in-vivo (chapter 4) and in-vitro (chapter 5) analyses, a number of questions arose that could not be answered by the neurosphere assay alone, and for which in-vivo experiments would not be appropriate, especially in the time-frame available to me. Two endogenous Fgfs, *Fgf10* and *Fgf18* show restricted expression to the ventromedial 3rd ventricle and are expressed by neurospheres, suggesting they may be regulators of progenitors within the ventromedial 3rd ventricle. In addition, neurospheres are responsive to NMDA, the selective agonist of the ionotropic glutamate receptor, NMDA-R. The increase in neurospherogenic efficiency in response to NMDA suggests neurogenic cells, alpha-tanycytes, proliferate in response to glutamatergic signalling. I therefore wished to determine whether FGF10, FGF18 and NMDA affect alpha-tanycytes.

My analyses (chapter 6) had revealed that (1) organotypic slice culture in collagen beds recapitulates the in-vivo environment, and (2) that treatment with FGF2 in this culture system increases alpha-tanycyte proliferation, as observed with FGF2 infusion in-vivo. Together, these results gave me confidence that this culture technique would be suitable to study the proliferative response of progenitor cells in response to FGF10, FGF18 and NMDA. I therefore aimed to use the organotypic slice culture assay to investigate the influence of local Fgfs on the hypothalamic niche, and further elucidate the response of niche cells to physiological stimuli.

Thus, in this chapter I use the organotypic slice culture assay to characterise the proliferative response of VZ cells of the ventromedial 3rd ventricle to FGF10 and FGF18. Furthermore, I determine whether alpha-tanycytes are responsive to stressors, including neuroexcitotoxicity and the adrenal steroids that mediate the physiological response to stress.

### 7.2.1: Alpha-tanycytes proliferate in response to FGF18

Several lines of evidence implicated endogenously expressed Fgf10 and Fgf18 in regulating proliferation and neurogenesis in tanycytes of the 3rd ventricle. Our previous studies showed *Fgf10* and *Fgf18* expression in restricted adult VZ regions, namely in ventral alpha2-tanycytes (*Fgf10/Fgf18*) and beta-tanycytes (*Fgf10*) (figure 1.9). In addition, we detected their expression in hypothalamic neurospheres (figure 3.4A). My analyses also revealed that supplementing neurosphere media with exogenous FGF10 and FGF18 differentially regulated the size and number of neurospheres. FGF10 increased the number of small neurospheres, while FGF18 generated an increase in large neurospheres, suggesting maintenance and proliferative actions respectively (chapter 5, figures 3.4B-C). The advantage of the slice culture assay in distinguishing proliferative responses of defined tanycyte and VZ regions led me to investigate whether exogenous administration of these Fgfs stimulated proliferation of tanycytes maintained in a niche, ex-vivo.

Previous in-vivo infusion and ex-vivo slice cultures had shown that FGF2 mediates a restricted proliferative response in alpha2-tanycytes (chapter 4 and 6). Addition of FGF2 to slice culture (SC) media therefore provided a positive control for alpha-tanycyte proliferation; SC media alone provided a baseline. Recapitulation of the experiments performed in chapter 6 showed that few PH3-positive cells were observed in SC media alone, compared to increased numbers in the alpha-tanycyte regions in response to FGF2, confirming these controls (figure 7.1A). In response to administration of FGF10, no increase in PH3-positive cells was seen: quantitative analyses showed no significant change from the control and a significant decrease in number compared to FGF2 (figure 7.1B). This result suggests FGF10 does not induce proliferation under these conditions, and may be acting to maintain an undifferentiated state and inhibit lineage progression as suggested by the neurosphere experiments.

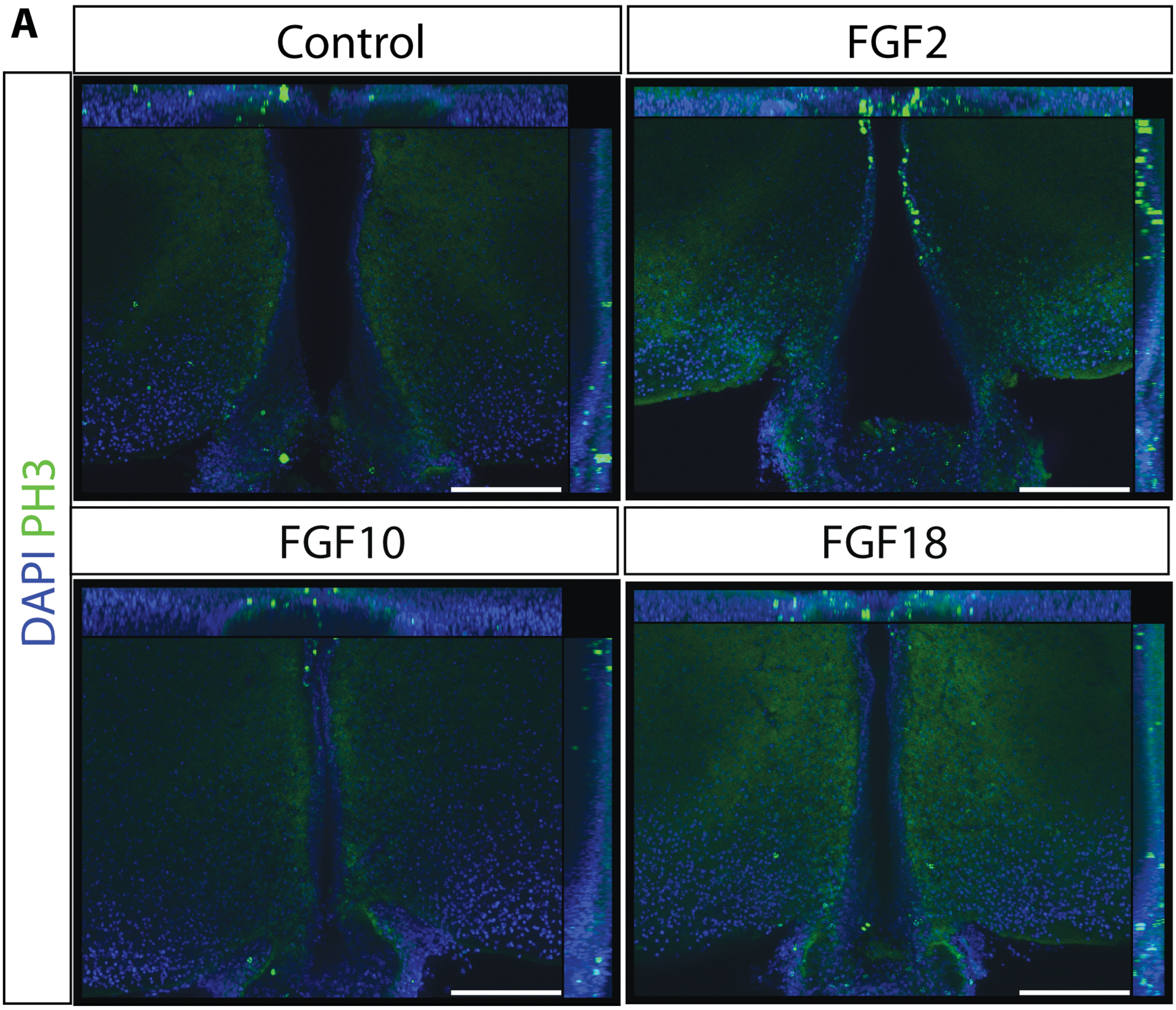
Similarly, although VZ PH3-positive cells were observed after addition of FGF18 (figure 7.1A), quantification revealed no significant difference from SC media

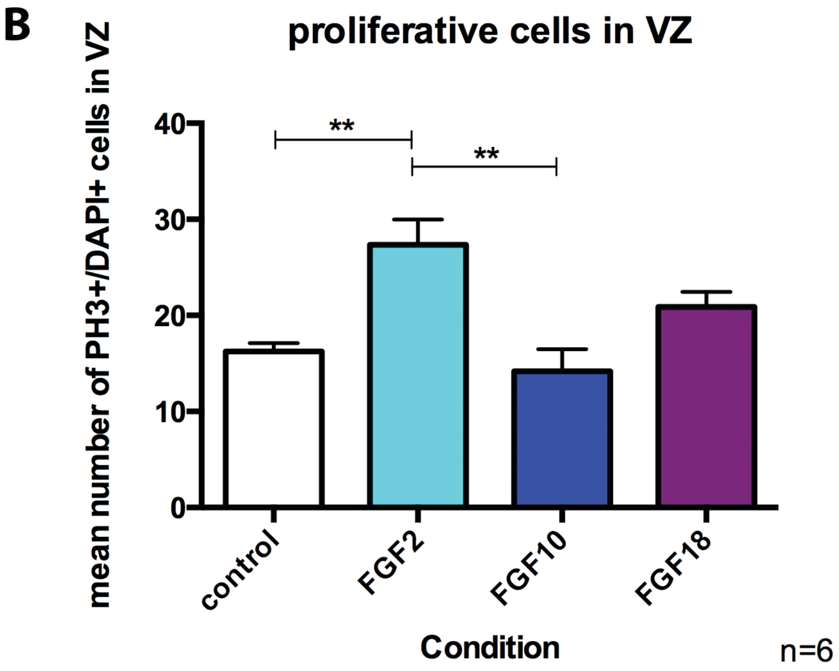
## Figure 7.1: Proliferative response to exogenously administered local Fgfs.

Tuberal hypothalamic slices were cultured in SC media for 24 hours, followed by culture in SC media alone (as control) or with the addition of FGF2, FGF10 or FGF18 (60ng/ml) to SC media for a further 24 hours.

A. MIPs of tuberal hypothalamic slices immunostained for PH3 expression (green), DAPI is used to label cell nuclei (blue). PH3 expression is upregulated in response to FGF2, while FGF10 and FGF18 addition does not appear to upregulate PH3 expression compared to control. Scale bar represents 100μm.

B. Mean number of PH3-positive cells in the VZ is quantified for the four conditions. A significant increase in PH3-positive is calculated in FGF2 media compared to control and FGF10 media (*p<0.05=**). A non-significant increase in PH3-positive cells is observed in FGF18 media compared to control and FGF10 media. Error bars represent SEM. (*n=6*).


A
Control
FGF2
FGF10
FGF18
DAPI PH3


B
proliferative cells in VZ
mean number of PH3+/DAPI+ cells in VZ
40
30
20
10
0
**
**
control
FGF2
FGF10
FGF18
Condition
n=6

alone (figure 7.1B). Despite no statistically significant increase in proliferative cells compared to SC media alone, a modest increase in PH3-positive cells was observed, above FGF10. This result suggests that FGF18 is capable of inducing proliferation, though is not as effective as FGF2. These results are in keeping with the neurosphere data that support FGF18 as able to enhance the size of neurospheres, but to do so minimally compared with FGF2.

In conclusion, no statistically significant change in proliferation was detected following the addition of exogenous FGF10 or FGF18 compared to SC media. An increase in PH3-positive cells was observed with the addition of FGF18, albeit not significant, supporting previous neurosphere data and potentially suggesting that FGF18 can induce proliferation in alpha-tanycytes. Increasing the number of analyses may result in statistically significant data. The lack of change in proliferation in FGF10 media compared to SC media suggests FGF10 is not mitogenic in this system.

### 7.2.2: Kainic acid induces alpha-tanycyte proliferation

Kainic acid (KA) is well-established as neuroexcitotoxic at high concentrations as it acts through the ionotropic glutamate receptor, the kainate receptor, to induce the same response as glutamate. High doses of KA lead to excessive excitatory neurotransmission, seizures and cytotoxicity (Routbort *et al.*, 1999). In the hypothalamus, administration of KA to membrane-interface cultures induces neuronal death of melanin concentrating-hormone immunoreactive (MCH) neurons and orexin neurons at concentrations of 30μM or above (Katsuki and Akaike, 2004). To test whether alpha-tanycytes can respond to neuronal death in the hypothalamus, tuberal hypothalamic slices were cultured with 50μM or 100μM KA compared to SC media alone.

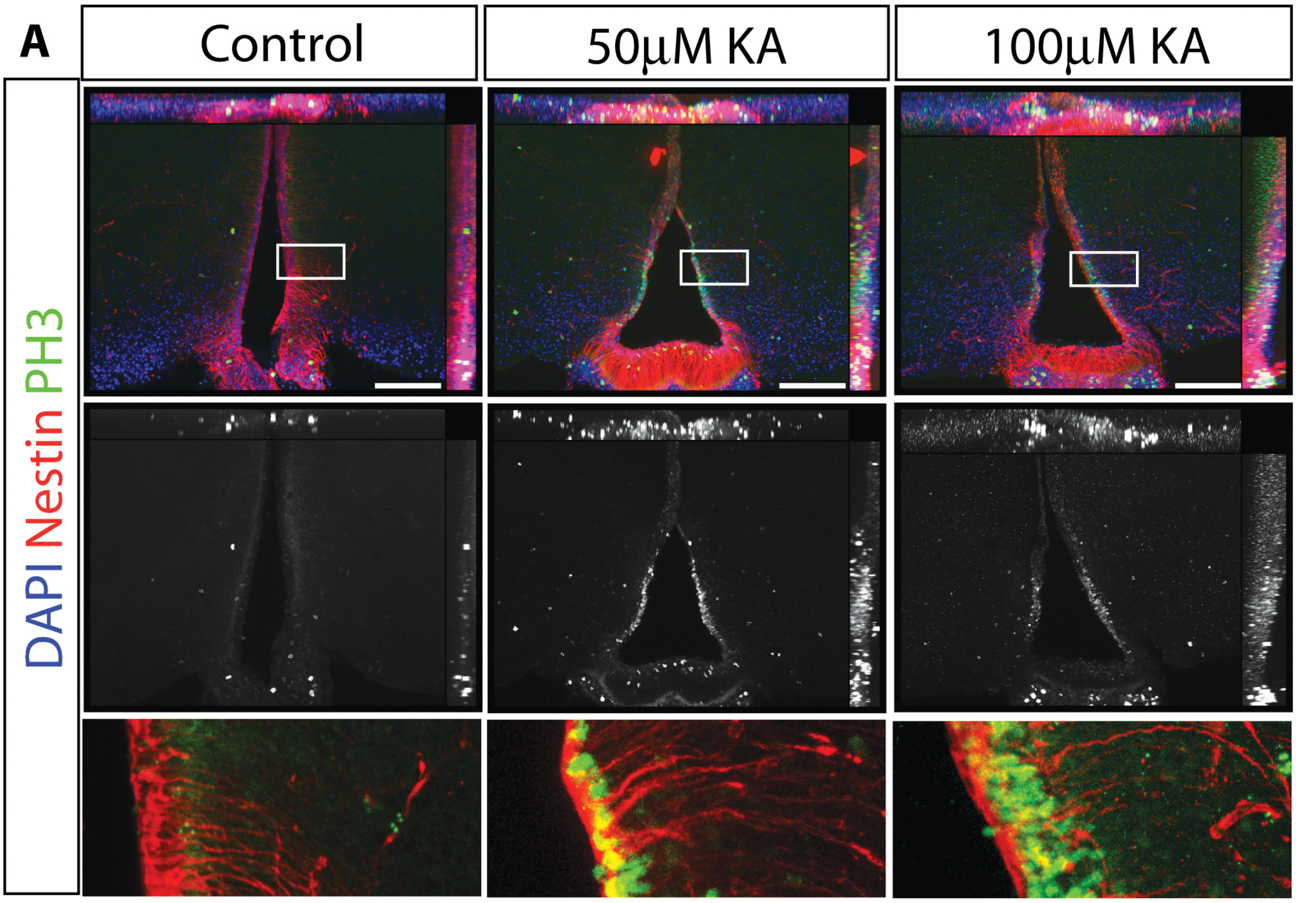
Addition of both concentrations of KA led to an observable increase in PH3-positive proliferating cells in the alpha-tanycyte region (figure 7.2A) compared to the control. Quantification of the number of PH3-positive cells in the VZ showed a statistically significant increase in the mean number of proliferative cells when

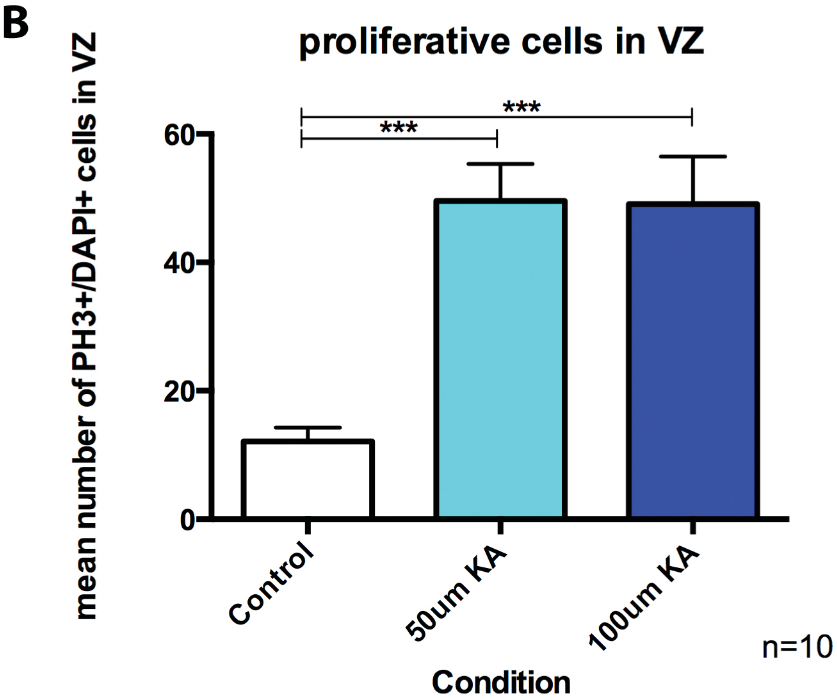
## Figure 7.2: Neuroexcitotoxic stimulus induces proliferation in alpha-tanycytes.

Increasing concentrations of kainic acid (KA) is added to SC media for 24 hours, following the 24-hour equilibration/recovery culture period. KA is added at concentrations of 50μM and 100μM to induce neuronal cell death by excitotoxicity, compared to SC media alone (control).

A. MIPs of tuberal hypothalamus immunostained for tanycyte cell marker, Nestin (red), and proliferative marker, PH3 (green, black/white). DAPI is used to mark cell nuclei (blue). Magnifications of the boxed regions are shown in bottom panel. An increase in PH3-positive cells is observed with the addition of KA, and can be seen to express Nestin. Scale bar represents 100μm.

B. Mean number of PH3-positive cells in VZ is quantified in the different conditions. A significant increase in the number of PH3-positive cells is calculated in KA treated media compared to control ($p<0.05=*$). No difference in the mean number of PH3-positive cells is observed between 50μM and 100μM KA treated media. Error bars represent SEM. ($n=10$).


A
Control
50μM KA
100μM KA
DAPI Nestin PH3


B
proliferative cells in VZ
mean number of PH3+/DAPI+ cells in VZ
60
40
20
0
***
***
Control
50um KA
100um KA
n=10
Condition

KA was added at both 50μM and 100μM concentrations (figure 7.2B). No difference was observed between the two concentrations, suggesting that receptor activation is saturated at 50μM KA and that the maximum proliferative response is achieved. PH3-positive cells were also observed parenchymally, suggesting widespread sporadic proliferation in response to excitotoxic concentrations of KA. However, a restricted region showing high levels of proliferation was observed in the alpha-tanycyte region, supporting alpha-tanycytes as responsive to neuronal death.

These data provide the first evidence that VZ cells, specifically alpha-tanycytes, proliferate in response to neuroexcitotoxic stimuli. Widespread proliferation can be observed throughout other regions of tissue, characteristic of KA induced neuronal death. Yet, the restricted proliferative response in the VZ is highly suggestive of a niche response to the death of neurons in hypothalamic nuclei, supporting alpha-tanycytes as receptive to physiologically stressful stimuli: death of neurons.

### 7.2.3: NMDA stimulates Fgf-dependent alpha-tanycyte proliferation

NMDA was similarly used in the neurosphere assay to determine whether neurospherogenic cells are responsive to excitatory neurotransmission, as a model of physiological stimuli (chapter 5). My neurosphere results indicated that proliferation increased in response to NMDA; however the responsive cell could not be reliably confirmed in that system. The slice culture assay, however, maintains progenitors in their niche and facilitates the distinction of proliferative cells by location. I therefore surmised that the slice culture assay might provide a valuable model in characterising the response to NMDA.

Analogous to KA, high concentrations of NMDA kill orexin and MCH neurons (Katsuki and Akaike, 2004); thus 50μM and 100μM NMDA was added to hypothalamic slices in tandem with the above experiment (figure 7.2). Both conditions led to an increase in PH3-positive cells in the alpha-tanycyte region,

## Figure 7.3: NMDA receptor activation upregulates proliferation via the Fgf signalling pathway.

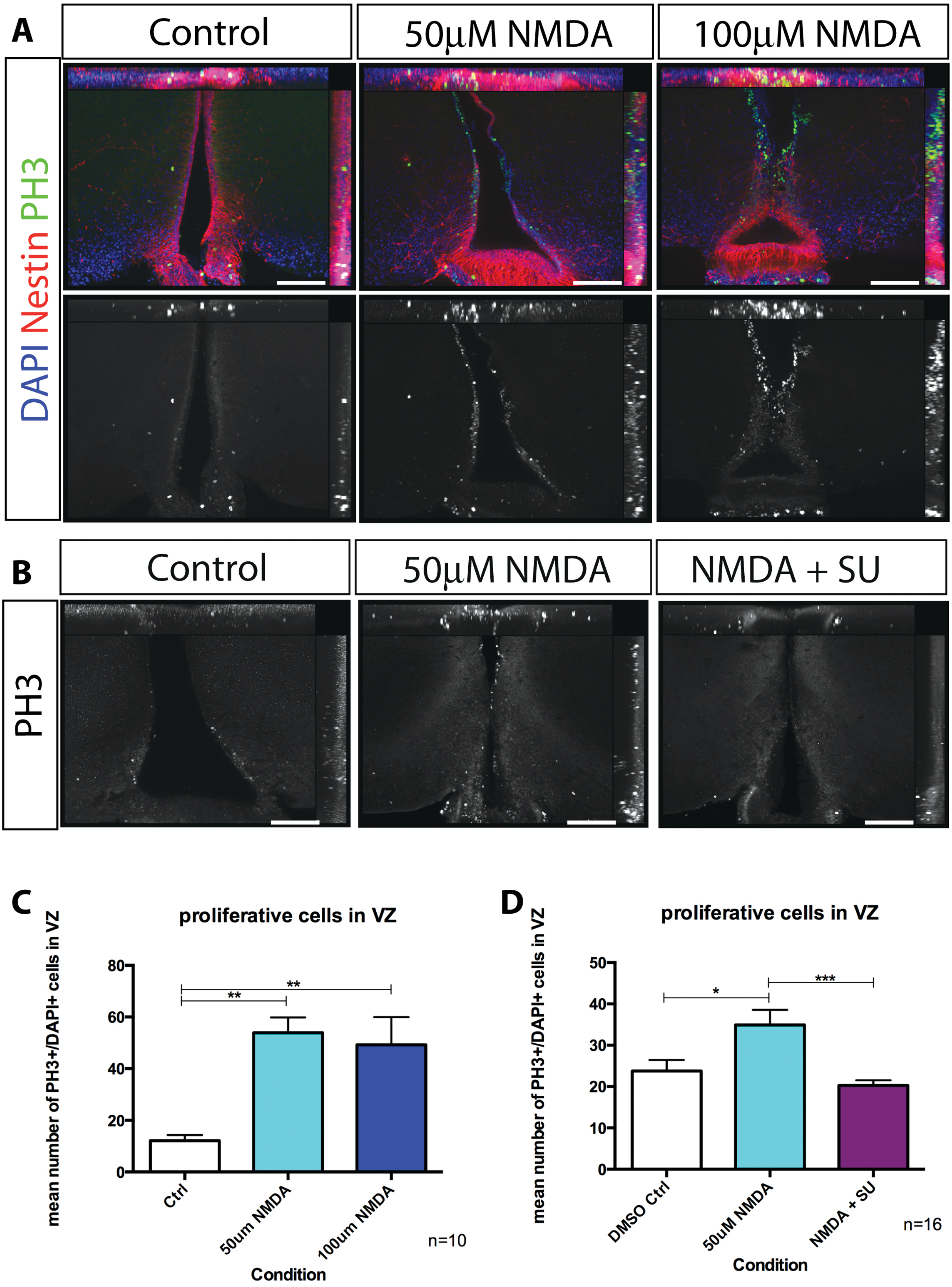
Increasing concentrations of N-methyl-d-aspartate (NMDA) is added to SC media for 24 hours, following the 24-hour equilibration/recovery culture period.

A. NMDA is added at concentrations of 50μM and 100μM to induce neuronal cell death by excitotoxicity, compared to SC media alone (control). These experiments were performed in tandem with the KA analysis, and the control is shared. MIPs of tuberal hypothalamus are immunostained for tanycyte marker, Nestin (red), and proliferative marker, PH3 (green, black/white). DAPI is used to stain cell nuclei (blue). An increase in PH3-positive cells is observed in NMDA treated media compared to control. Scale bar represents 100μm.

B. 50μM NMDA is added to SC media, compared to 20μM SU5402 with 50μM NMDA. DMSO is added accordingly. MIPs are shown for tuberal hypothalamic slices immunostained for proliferative marker, PH3. DMSO controls have PH3-positive cells in VZ, as do NMDA treated media. Low numbers of PH3-positive cells are observed with the addition of SU5402. Scale bar represents 100μm.

C. Mean number of proliferative cells in VZ is quantified for the different conditions: control, 50μM NMDA, 100μM NMDA. A significant increase is calculated in the number of PH3-positive cells in 50μM and 100μM NMDA treated media compared to control media ($p<0.05=*$). No difference is seen in the mean number of PH3-positive cells between 50μM and 100μM NMDA treated media. Error bars represent SEM. ($n=10$).

D. Mean number of proliferative cells in VZ is quantified for the different conditions: DMSO control, 50μM NMDA (+DMSO), 50μM NMDA + 20μM SU5402. A significant increase in the number of PH3-positive cells is calculated in 50μM NMDA treated media compared to control. A significant decrease in the number of PH3-positive cells is calculated when 20μM SU5402 is added to NMDA treated media ($p<0.05=*$). No statistical difference is calculated between the control and SU5402 treated media. Error bars represent SEM. ($n=16$).


A
Control
50μM NMDA
100μM NMDA
DAPI Nestin PH3
B
Control
50μM NMDA
NMDA + SU
PH3
C
proliferative cells in VZ
mean number of PH3+/DAPI+ cells in VZ
80
60
40
20
0
**
**
Ctrl
50um NMDA
100um NMDA
Condition
n=10
D
proliferative cells in VZ
mean number of PH3+/DAPI+ cells in VZ
50
40
30
20
10
0
*
***
DMSO Ctrl
50uM NMDA
NMDA + SU
Condition
n=16

similarly to KA (figure 7.3A). However, in contrast, few proliferative cells were observed parenchymally, suggesting that 24-hours of NMDA exposure does not elicit the widespread proliferation characteristic of neuroexcitotoxic stimuli, as seen with KA. Instead, neuronal death may be restricted to a specific nuclei or neuronal type. Quantification of PH3-positive cells revealed a statistically significant increase in mean number of proliferating VZ cells in response to both NMDA concentrations compared to control (figure 7.3C). Again, no statistical difference was observed between 50μM and 100μM NMDA, suggesting that 50μM is sufficient to induce the maximum proliferative response.

While 50μM NMDA is neuroexcitotoxic in hypothalamic slice cultures, the lack of proliferation throughout parenchymal regions, compared to the specific proliferation in the VZ, suggests that the proliferation is a direct response to NMDA rather than a response to neuronal death. As FGF2 stimulates alpha-tanycyte proliferation, the Fgf receptor inhibitor, SU5402, was added to NMDA-supplemented media to determine whether NMDA induced-proliferation was dependent on Fgf signalling. While 50μM NMDA induced VZ proliferation, addition of SU5402 inhibited this effect (figure 7.3B). Quantification of the mean number of PH3-positive cells revealed a statistically significant decrease in PH3-positive cells with the addition of SU5402, confirming NMDA-induced proliferation as Fgf-dependent (figure 7.3D).

These data support the idea that alpha-tanycytes proliferate in response to NMDA, and suggest that alpha-tanycytes are directly responsive to NMDA. Furthermore, given that addition of SU5402 to hypothalamic slices inhibits this response, the proliferation appears to be regulated by Fgf signalling. These results confirm and extend the neurosphere analysis by indicating that VZ cells are responsive to NMDA, rather than being a fully differentiated population of cells. These results provide evidence that hypothalamic progenitor/stem cells proliferate in response to physiological stimuli modelled by isotropic glutamate receptor activation.

### 7.2.4: Glucocorticoid receptors are expressed on VZ cells and are increased in response to dexamethasone

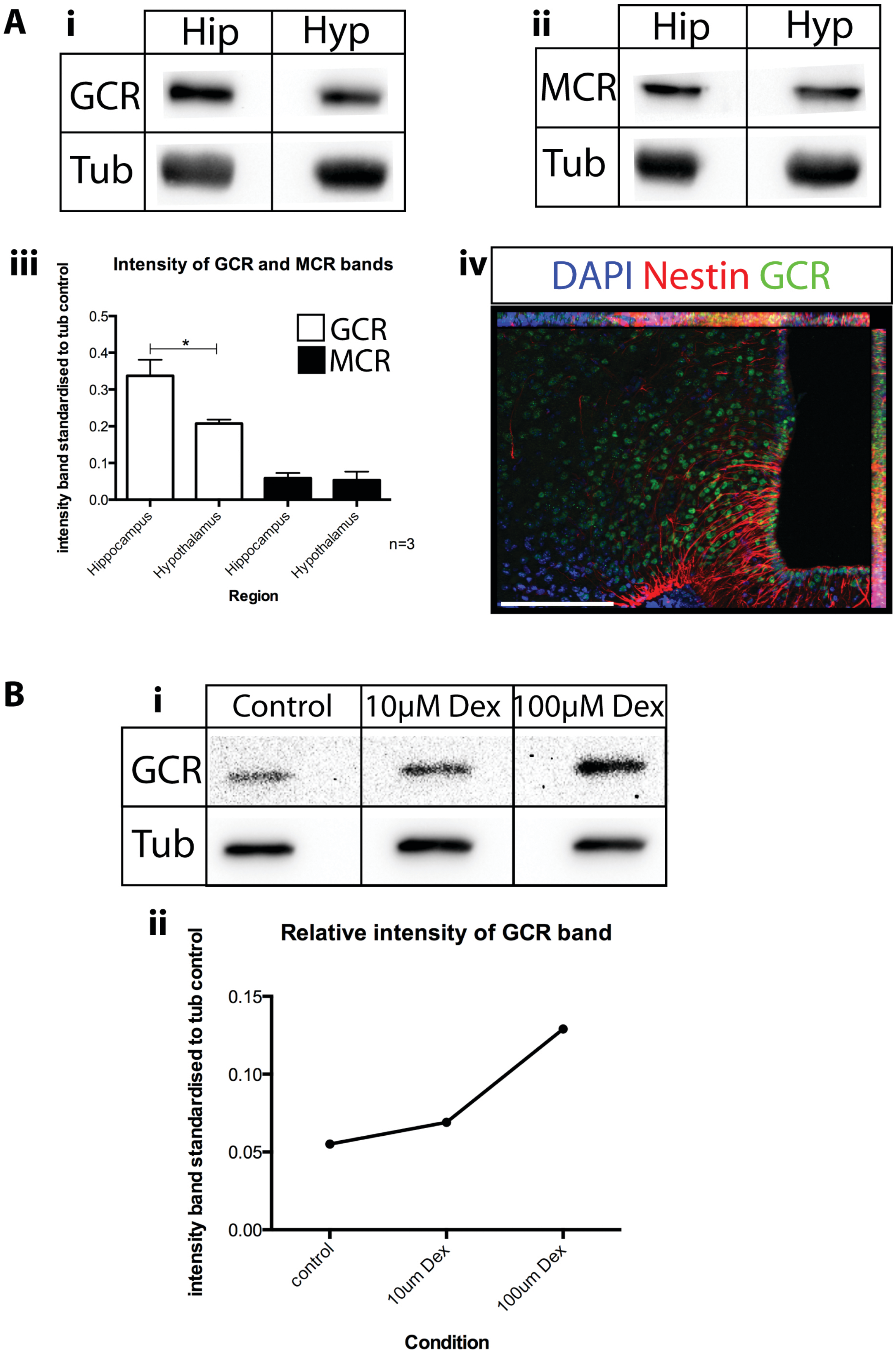
The hypothalamus regulates the physiological response to stress, mediated through activation of mineralocorticoid receptors (MCR) and glucocorticoid receptors (GCR), activated by corticosterone in rodents (Herman, 2013; Busnardo *et al.*, 2012). The established view suggests that MCRs regulate an immediate sympathetic nervous system-response to stress, while the lesser-characterised long term adaptive response to stress is regulated by GCRs (De Kloet, 2003). Activation of each receptor by the stress hormone results in further upregulaton of the receptor, ensuring an increase in corticosterone is sequestered. Both receptors are ubiquitously expressed in the hippocampus, an area that is particularly sensitive to changes in stress (Gould and Tanapat, 1999; De Kloet, 2003). In order to examine the presence of GCRs in the hypothalamus, tissue lysates were prepared and western blots were performed to detect expression of the respective receptor proteins in the hippocampus, as a control, and the hypothalamus (figure 7.4A).

Western blots revealed GCR and MCR expression in both the hippocampus and the hypothalamus (figure 7.4Ai and ii respectively). When the relative intensity of the protein bands was compared to β-tubulin controls, a statistically significant decrease in the amount of GCR was found in the hypothalamus compared to the hippocampus (figure 7.4Aiii). In contrast, no change was observed in the relative amounts of MCR between the two regions. These data indicate that both receptors are present in the hypothalamus, but show that at the time of tissue lysate preparation, GCR expression is lower in the hypothalamus than in the hippocampus. To confirm GCR expression in the hypothalamus, the antibody used for western blotting was used for immunolabelling of hypothalamic sections (figure 7.4Aiv). GCR was detected on VZ cells, many of which could be co-labelled with the tanycyte marker, Nestin (note, GCR was additionally detected on hypothalamic parenchymal cells). This result supports the established view that GCR is expressed in stress-sensitive

## Figure 7.4: Glucocorticoid receptor is expressed by tanycytes and is upregulated in response to dexamethasone.

A. Lysates of hippocampus and hypothalamus are prepared and western blots are performed to identify glucocorticoid receptor (GCR) and mineralocorticoid receptor (MCR). β-tubulin expression is used as a control.
   i. GCR is present in hippocampal and hypothalamus lysates
   ii. MCR is present in hippocampal and hypothalamus lysates
   iii. The relative intensity of protein bands is standardised to β-tubulin controls for 3 repeats. A significant decrease in the amount of GCR is calculated in the hypothalamus compared to the hippocampus. No statistical difference is calculated in MCR levels between the hippocampus and the hypothalamus. Error bars represent SEM. (*n=3*).
   iv. MIP of tuberal hypothalamus is shown, immunostained for tanycyte marker, Nestin (red), and GCR (green). DAPI is used to stain cell nuclei. Parenchymal cells, VZ cells and Nestin-positive tanycytes express GCR. Scale bar represents 100μm.

B. Synthetic glucocorticoid agonist, dexamethasone (Dex), is added to SC media at 10μM and 100μM for 24-hours following the equilibration/recovery period.
   i. Lysates of hypothalamic slices are prepared and western blots are performed to identify semi-quantitative levels of GCR. β-Tubulin expression is used as a control. An increase in strength of the GCR protein band is observed with increasing concentration of Dex.
   ii. The relative intensity of GCR protein bands is standardised to β-Tubulin controls. An increase in intensity is observed with increasing concentration of Dex.


A
i
Hip
Hyp
GCR
Tub
ii
Hip
Hyp
MCR
Tub
iii
Intensity of GCR and MCR bands
intensity band standardised to tub control
0.5
0.4
0.3
0.2
0.1
0.0
*
GCR
MCR
Hippocampus
Hypothalamus
Hippocampus
Hypothalamus
n=3
Region
iv
DAPI Nestin GCR
B
i
Control
10µM Dex
100µM Dex
GCR
Tub
ii
Relative intensity of GCR band
intensity band standardised to tub control
0.15
0.10
0.05
0.00
control
10um Dex
100um Dex
Condition

regions of the brain, and reveals that GCR is expressed on tanycytes, suggesting that tanycytes are responsive to stress hormones.

Dexamethasone is a synthetic and specific activator of the glucocorticoid receptor, and is thus frequently used to study the downstream effects of GCR activation (Snyder *et al.*, 2011; Mulholland *et al.*, 2005). I therefore next cultured tuberal hypothalamic slices in SC media alone or with the addition of 10μM or 100μM dexamethasone (Dex). After culture, lysates of hypothalamic slices were prepared, and western blots performed to determine whether Dex increases GCR expression, providing evidence of a physiological response to GCR activation (figure 7.4B). An increase in GCR expression was observed after addition of 10μM Dex; this increased further in response to 100μM Dex (figure 7.4Bi). Standardising the relative intensity of receptor bands to β-tubulin controls confirmed a subtle increase in response to 10μM Dex and a large increase in GCR expression in response to 100μM Dex (figure 7.4Bii). These results indicate that the physiological response to GCR activation is maintained in slice culture, and that addition of 100μM Dex results in a large, measurable response, suitable for further studies.

Together these data confirm the expression of GCR in the hypothalamus, and provide evidence of its expression on tanycytes. In addition, they reveal an increase in receptor expression in response to activation. These results suggest that 100μM Dex is a suitable concentration to further investigate the downstream response to GCR activation in hypothalamic slice cultures.

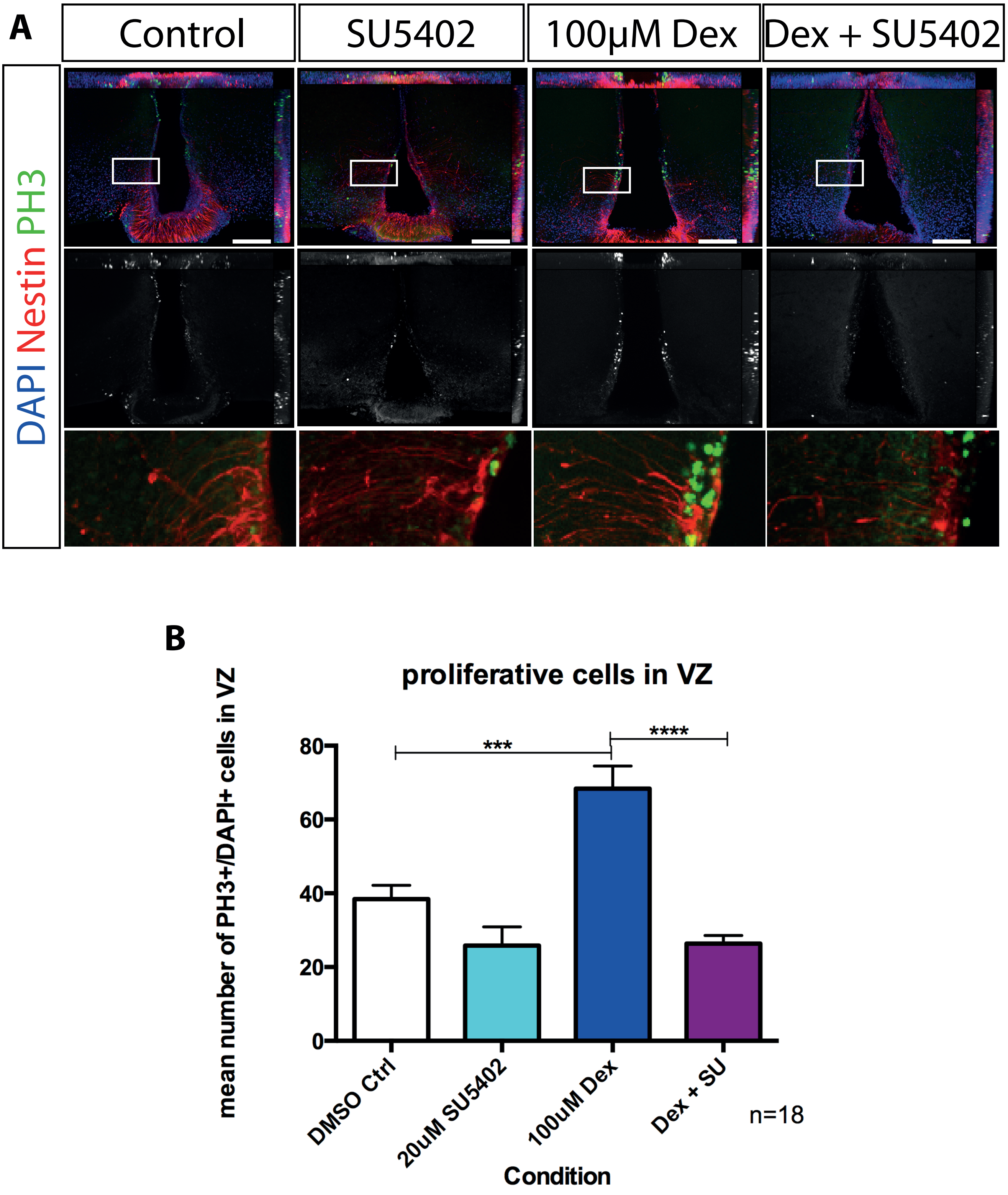
### 7.2.5: Dexamethasone induces Fgf-dependent proliferation in alpha-tanycytes

In order to investigate whether hypothalamic niche cells proliferate in response to GCR activation, hypothalamic slices were cultured in SC media alone (with DMSO) or in media supplemented with Dex, or the Fgf receptor inhibitor, SU5402, or both (figure 7.5).

## Figure 7.5: Dexamethasone stimulates Fgf dependent proliferation in alpha-tanycytes.

Hypothalamic slices are cultured for the 24-hour equilibration/recovery period, followed by 24-hour culture in experimental conditions: DMSO control, 20μM SU5402, 100μM Dex (+DMSO), 100μM Dex + 20μM SU5402.

A. MIPs are shown of hypothalamic tuberal slices immunostained for tanycyte marker, Nestin (red), and proliferative marker, PH3 (green, black/white). Low numbers of PH3-positive cells are observed in SU5402 treated media. PH3-positive cells can be observed in DMSO control media, while high number of proliferative cells can be seen in alpha-tanycyte regions in Dex treated media. PH3-positive cells co-label with Nestin (bottom panel). Scale bars represent 100μm.
B. Mean number of PH3-positive cells in VZ is quantified in the different conditions. A significant increase in the number of PH3-positive cells is calculated in Dex treated media alone compared to DMSO control and media containing Dex with SU5402 ($p<0.05=*$). No statistical difference in the mean number of PH3-positive cells can be calculated between DMSO control and media containing SU5402. Error bars represent SEM. ($n=18$).


A
Control
SU5402
100μM Dex
Dex + SU5402
DAPI Nestin PH3
B
proliferative cells in VZ
mean number of PH3+/DAPI+ cells in VZ
80
60
40
20
0
***
****
DMSO Ctrl
20uM SU5402
100uM Dex
Dex + SU
n=18
Condition

PH3-positive cells were observed in the VZ of all conditions, with a particular increase in proliferative cells in media supplemented with 100μM Dex (figure 7.5A). Addition of SU5402 to Dex supplemented media reduced the increase in PH3-positive cells. High power analyses showed that PH3-positive cells co-localised with Nestin-positive cells in the alpha-tanycyte domain. Quantification of PH3-positive cells revealed a statistically significant increase in the mean number of proliferative cells in response to Dex compared to SC media controls, indicating that GCR activation results in proliferation of alpha-tanycytes (figure 7.5B). Addition of the Fgf receptor inhibitor, SU5402, to Dex-supplemented media, resulted in a statistically significant decrease in the mean number of PH3-positive cells, providing evidence that the proliferative response to GCR activation is mediated by Fgf signalling.

In conclusion, these hypothalamic slice culture data provide evidence that alpha-tanycytes are responsive to physiological stimuli including neuronal death and excitatory neurotransmitters. In addition, specific activation of GCR by Dex, modelling corticosterone activation of the receptor, results in a proliferative response of alpha-tanycytes, supporting a role for these niche cells in the long-term adaptation to stress. The finding that the proliferative response to physiological stimuli is mediated by the Fgf signalling pathway is in agreement with our previous data that propose Fgf signalling as a core regulator of alpha-tanycyte proliferation, both in-vivo and in-vitro (Robins *et al.*, 2013a). Such regulation may be performed by endogenous Fgf18, shown to modestly increase proliferation, and/or by upregulating endogenous Fgf2 expression, shown to significantly increase proliferation. The hypothalamic slice culture assay thus provides invaluable data in focusing further investigation into the alpha-tanycyte response to physiological stimuli, with specific attention on the stress response.

## 7.3: Discussion

My previous studies have identified alpha2-tanycytes as component cells of a neural stem/progenitor cell niche in the ventromedial hypothalamus. This evidence is provided by neural stem cell marker profiling, BrdU incorporation and retention assays, lineage-tracing analyses of transgenic animals and neurosphere assays. A combination of techniques is required to mutually support results from alternate perspectives, and to interrogate systems based on molecular, cellular, tissue and organism contexts.

While neurosphere assays are a valuable model for molecular and cellular regulatory questions, and in-vivo experiments are the gold-standard for modeling true biological responses, technical limitations exist for both. Neurosphere assays are primary cultures of cells, removed from their environment and supplemented with high levels of mitogens, and thus giving minimal information about the response of distinct cell types within a neural stem cell niche (Jensen and Parmar, 2006; Pastrana *et al.*, 2011). In contrast, in-vivo experiments can be used to interrogate progenitor cells individually, within a niche or in the context of the entire organism (Bonaguidi *et al.*, 2012). However, the expense of founding and maintaining transgenic mice lines, as well as ensuring experiments are performed within legal frameworks, results in costly and lengthy studies. The organotypic slice culture assay, however, provides an intermediate between neurosphere assays and in-vivo experiments in studying niche cells (Gahwiler *et al.*, 1997), confirming and extending previous work, and focussing future work.

Here, I will discuss results from slice culture experiments that aimed to further our understanding of the regulatory role of local Fgfs on tanycyte niche cells. In addition, I will argue that the slice culture assay provides evidence that alpha-tanycytes undergo Fgf-dependent proliferation in response to stress stimuli.

### 7.3.1: Fgf regulation of alpha-tanycyte proliferation

Previous analyses in Placzek lab have characterised the expression of different Fgfs in the ventromedial hypothalamus, identifying *Fgf10* and *Fgf18* to be expressed in a restricted expression pattern within the VZ of the 3$^{rd}$ ventricle. Specifically, *Fgf10* is expressed by beta- and alpha2-tanycytes, while *Fgf18* expression is restricted to alpha2-tanycytes alone. In addition, recent studies have implicated Fgf10-positive tanycytes in neurogenesis in response to dietary changes (Haan *et al.*, 2013). Studies by Sarah Robins identified *Fgf10* and *Fgf18* expression in neurospheres, and my studies in which neurosphere media was supplemented with FGF10 or FGF18, suggest roles in neural progenitor maintenance and proliferation respectively (figure 5.4). In order to elucidate the proposed role of Fgf18 on progenitor proliferation, similar to the effect of FGF2, FGF ligands were added to slice culture (SC) media and PH3-positive cells were analysed as a measure of proliferation (figure 7.1)

In agreement with FGF2-infusion in-vivo (chapter 4) and FGF2 treated slice cultures (chapter 6), FGF2 supplemented SC media results in an observable increase in PH3-positive cells in VZ of the alpha-tanycyte domain (figure 7.1A). In contrast, addition of FGF10 does not result in an observable proliferative response that can be distinguished from SC media alone. Addition of FGF18, similarly, does not lead to a significant change in proliferation compared to SC media. When the numbers of PH3-positive cells are quantified, however, a statistically non-significant but modest increase in VZ proliferation is observed in FGF18 supplemented SC media compared to SC media alone (figure 7.1B). Addition of FGF2 results in a significant increase in proliferation, while addition of FGF10 has no effect on the mean number of PH3-positive cells compared to SC media alone. These results support FGF2 as having a significant mitogenic role on alpha-tanycytes, while FGF10 does not influence proliferation. The small increase in VZ proliferation in response to FGF18 does support a role for this local ligand in regulating alpha-tanycyte proliferation.

These results support the in-vitro neurosphere data that indicate Fgf signalling is required for neurospherogenesis, and highlight FGF2 as a substantial mitogen, generating large neurospheres through proliferation. These data similarly show alpha-tanycytes, the neurospherogenic cell, as highly proliferative in response to FGF2. The neurosphere assay also suggests that Fgf10 acts to maintain neurospherogenic cells as uncommitted quiescent cells, as shown by the comparatively high number of small neurospheres in response to FGF10 (figure 5.4B-C). The slice culture data supports this possibility as no change in proliferation is observed compared to controls (figure 7.1B). This particular slice culture experiment does not extend this observation as the experiment is designed to analyse proliferation, and not quiescence. Future slice culture assays could interrogate the cell-cycle dynamics of alpha-tanycytes to determine whether Fgf10 maintains cells at $G_0$ phase, associated with quiescence (Salomoni and Calegari, 2010). In addition, the neurosphere assay suggests FGF18 can increase proliferation of neurospherogenic cells, similarly to FGF2 but with reduced potency. Supplementing SC media with FGF18 results in a small increase in proliferation, analagous to neurosphere results, that is not significantly different from SC media alone or SC media supplemented with FGF2. This result supports local Fgf18 signalling in regulating alpha-tanycyte proliferation.

The comparable difference in proliferation in response to FGF2 and FGF18, analysed by neurospheres and organotypic slice cultures, suggests their roles may be distinct. VZ cells of the 3rd ventricle express *Fgf2* (Gonzalez *et al.*, 1994), while *Fgf18* expression is restricted to alpha2-tanycytes. As FGF2 is a potent stimulator of proliferation, this suggests *Fgf2* expression may be under tight control and is increased in response to particular stimuli to evoke an increase in proliferation, and potentially neurogenesis. In contrast, local *Fgf18* expression may be required to maintain low levels of constitutive proliferation and neurogenesis, as observed in the unchallenged hypothalamus (Kokoeva *et al.*, 2007). These two neurogenic responses are distinct, with high levels being associated with a particular stimuli and a shift in neural circuitry to maintain physiological homeostasis, and low levels being associated with maintaining tissue homeostasis including replacement of old/dying neurons. Indeed, *Fgf2* expression in neurogenic regions is increased in response to physiological stimuli including running (Gomez-Pinilla *et al.*, 1997) and brain insults such as stroke (Yoshimura *et al.*, 2001).

The statistically non-significant increase in the mean number of PH3-positive cells in response to exogenous FGF18 may also be a result of the actions of the endogenous ligand. Proliferation occurs in SC media alone, some of which may be attributed to endogenous expression of *Fgf18*, thus the true response to

Fgf18 cannot be determined by exogenous administration alone. The slice culture assay provides a tool for future research, in which viral transfections to knockout endogenous *Fgf* expression in tanycytes can be combined with the exogenous ligand, to characterise the required role of endogenous Fgf18 and/or Fgf10.

An important consideration is that while the restricted expression of *Fgf10* and *Fgf18* to neurospherogenic cells may implicate a role in progenitor regulation, their expression in this region may be required for roles that are not attributed to the niche. Tanycytes have an array of functions including hormone release regulation and blood brain barrier roles, for which Fgf10 and Fgf18 may have unknown requirements. However, considering the roles of Fgf10 and Fgf18 in development and progenitor proliferation in other tissues (Sahara and O'Leary, 2009; Haque *et al.*, 2007), it supports a role for progenitor regulation in this brain region. In addition, Neurosphere assays and slice culture assays do suggest a role for Fgf10 and Fgf18 in the maintenance of the 3rd ventricular niche. The alpha-tanycyte response to FGF2, however, overshadows the subtle proliferative changes that occur in response to FGF18, and thus may dissuade further investigation despite its potential importance.

In summary, the organotypic slice culture assays here confirm previous neurosphere data, and indicate that alpha-tanycytes do proliferate in response to FGF2 and FGF18. The lack of a change in proliferation in response to FGF10 supports the proposed idea that Fgf10 may maintain progenitors as uncommitted quiescent cells, or may suggest no regulation upon the niche. The role of Fgf10 is particularly pertinent as recent studies use conditionally inducible Fgf10::CreER$^{T2}$ mice to study tanycyte neurogenesis, thus in the absence of a defined role for Fgf10 in the niche, the value of this model cannot be assured and its use may lead to inconsistencies between studies. Despite this limitation, it is evident that alpha-tanycytes are highly regulated by Fgf signalling.

### 7.3.2: Alpha-tanycytes proliferate in response to neuroexcitotoxic stimuli

Excitotoxicity is a well characterised process of neuronal cell death, in which excessive glutamatergic signalling via NMDA, AMPA and kainate receptors, leads to prolonged receptor activation, non-transitory neuronal depolarisation, sustained high intracellular-calcium levels and neuronal death (Sattler and Tymianski, 2001). Such events occur in response to pathological conditions including stroke and epilepsy (Marini *et al.*, 2007), and are contributing factors to the progression of many neurodegenerative disorders including Parkinson's and Huntington's disease (Mehta *et al.*, 2013). Similarly, neurogenesis is enhanced in response to stroke and seizure (Yoshimura *et al.*, 2001; Yoshimura *et al.*, 2003), while dysregulation of neurogenesis is implicated in the aetiology of Parkinson's (section 1.1.2, page 25). Furthermore, administration of kainic acid (KA) as a model of temporal lobe epilepsy has been shown to increase proliferation in Hes5::GFP-positive neural stem cells in the hippocampus, providing evidence that the neurogenic response to an excitotoxic insult occurs at the level of the neural stem cell (Lugert *et al.*, 2010). In order to determine whether hypothalamic alpha-tanycytes, the neural stem/progenitor cell of the 3rd ventricular niche, respond to excitotoxic insult, similarly to neural stem cells in the classically defined SVZ and SGZ of the adult central nervous system, hypothalamic slices were cultured with the addition of excitotoxic stimuli, KA and NMDA.

Previous studies have shown that supplementing hypothalamic cultures with KA at concentrations of 30μM or above results in the loss of orexin and melanin-concentrating hormone (MCH)-positive neurons in the lateral hypothalamus, confirming its excitotoxic actions in slice culture (Katsuki and Akaike, 2004). I therefore cultured hypothalamic slices in SC media alone or with the addition of 50μM or 100μM KA to observe whether excitotoxic concentrations stimulated a proliferative response of alpha-tanycytes (figure 7.2). 24-hour incubation with both concentrations was sufficient to induce a proliferative response in alpha-tanycytes that was statistically significant, confirming that alpha-tanycytes do proliferate in response to excitotoxic stimuli, similarly to neural stem cells in the SGZ. The lack of change in proliferation between the two concentrations also suggests 50μM KA saturates the response, resulting in the maximum proliferation capable in the VZ. Furthermore, PH3-positive cells are observed adjacent to the 3rd ventricle and in the surrounding parenchyma, which appear to increase at higher concentrations of KA. Such proliferation is characteristic of activation of parenchymal microglial cells in response to excitotoxic insult (Rogove *et al.*, 2002). This result supports alpha-tanycytes as sensitive to neuronal death, and thus suggests alpha-tanycytes can respond to hypothalamic insults to replace lost neurons.

In tandem with the above experiment, NMDA was also added at concentrations of 50μM and 100μM, previously shown to induce a selective loss of orexin neurons in hypothalamic slice cultures (Katsuki and Akaike, 2004). Analogous to KA results, 50μM NMDA is sufficient to induce a proliferative response in the VZ of the alpha-tanycyte domain (figure 7.3A/C). Again, no difference is calculated in the quantified number of PH3-positive cells between concentrations, indicating that the maximum proliferative response is stimulated by 50μM NMDA. These results suggest alpha-tanycytes respond to NMDA induced excitotoxicity. Alternatively, considering the NMDA results in a particularly selective loss of lateral hypothalamic neurons, and parenchymal proliferation does not appear to be high, compared to KA treated slices, these data also suggest alpha-tanycytes are directly sensitive to glutamatergic signalling. In support of this conclusion, culturing neurospheres in 10μM NMDA is sufficient to increase neurospherogenic efficiency (figure 5.5). This NMDA stimulated increase in proliferation in the absence of neuronal cell death in the neurosphere assay, coupled with an increase alpha-tanycyte domain PH3-positive VZ cells in hypothalamic culture, supports neural stem/progenitor cells of the 3rd ventricle as sensitive to excitatory neurotransmission. This conclusion is in agreement with a number of studies that have identified increased proliferation in response to glutamatergic signalling in the SGZ and SVZ (Xiao *et al.*, 2013; Brazel *et al.*, 2005b), and evidence that radial glia cells express NMDA receptors (Muth-Kohne *et al.*, 2010).

Taken together these results support alpha-tanycyte proliferation in response to excitotoxic stimuli, and support alpha-tanycytes as directly responsive to glutamatergic signalling. As both NMDA and KA receptors are activated by glutamatergic signalling, the proliferative response observed by alpha-tanycytes in response to KA treatment may be a combination of direct stimulation of receptors on progenitors as well as a response to excitotoxic insult.

Interestingly, when KA and NMDA were previously added to hypothalamic slices to induce neuronal death, following a 17-day culture period, the authors observed a recovery of neurons 72-hours after the excitotoxic insult, suggesting new neurons replace those that are lost (Katsuki and Akaike, 2004). Although 72-hours is unlikely to be sufficient time to generate neurons from the 3rd ventricular niche, it supports a neurogenic mechanism in the hypothalamus that is sensitive to neuronal death. As these data provide evidence of increased alpha-tanycyte proliferation in response to KA and NMDA treatment, alpha-tanycytes may contribute to the long-term recovery of neural networks.

These data implicate alpha-tanycytes as responsive to physiological effectors and pathological stimuli, in a manner that recapitulates FGF2 treatment. As such, the Fgf receptor inhibitor, SU5402, was added to cultures containing 50$\mu$M NMDA to determine whether Fgf signalling regulates the response to physiological stimuli (figure 7.3B/D). Indeed, preventing Fgf receptor phosphorylation and down stream signalling inhibits the proliferative response of VZ cells to NMDA receptor activation. This result indicates alpha-tanycyte proliferation in response to glutamatergic signalling is Fgf dependent, and that Fgf signalling provides the major instructive pathway for proliferation in the 3rd ventricular niche. Alternatively, high concentrations of NMDA and subsequent excitotoxicity may upregulate *Fgf* expression leading to proliferation, independently of NMDA receptor activation in progenitors. However, considering the prevailing view that neural stem/progenitor cells express ionotropic glutamate receptors in classically neurogenic regions (Brazel *et al.*, 2005b; Xiao *et al.*, 2013), these data support a similar mechanism in the 3rd ventricular niche.

In conclusion, culturing organotypic hypothalamic slices with ionotropic glutamate receptor agonists, NMDA and KA, results in alpha-tanycyte proliferation. In addition, NMDA induced alpha-tanycyte proliferation is Fgf dependent, further highlighting Fgf signalling as a major regulator of alpha-tanycyte proliferation. Future studies should use reduced concentrations of NMDA, in slice culture specifically, to distinguish between excitotoxic insult and direct receptor activation of progenitors.

### 7.3.3: Glucocorticoid receptor activation induces Fgf dependent proliferation in alpha-tanycytes

The data above provide evidence that alpha-tanycytes can respond to extreme tissue stress such as excitotoxicity, and that the proliferative response to glutamatergic signalling is regulated by Fgf signalling. Recent efforts to elucidate the physiological implication of neurogenesis from the hypothalamic 3rd ventricle have identified a complex involvement in energy-balance. Studies have found that CNTF-induced hypothalamic proliferation results in maintained weight-loss in adult mice (Kokoeva *et al.*, 2005), while, seemingly contradictory, destruction of newborn hypothalamic cells leads to increased energy expenditure (Lee *et al.*, 2012a). Although the role of progenitors in energy-balance is of interest in current society, and is likely to be financially lucrative in the future, the complex and often contradictory reports suggest an incomplete framework in which experiments are designed.

My perspective of the physiological implications requires consideration of the significance of hypothalamic proliferation and/or neurogenesis on an initially broader scale. Energy-balance is one important homeostatic function of the hypothalamus, and one that I believe is closely associated with environmental stress. The hypothalamus responds to environmental stress and challenges such as changes in food abundance, temperature and seasons, thus the hypothalamus responds to stressors to maintain homeostasis. In agreement, the stress response is regulated by the hypothalamic-pituitary-adrenal (HPA) axis (Herman, 2013). Briefly, the hypothalamus releases corticotropin-releasing hormone (CRH) that acts on the pituitary to stimulate release of adrenocorticotropic hormone (ACTH). ACTH stimulates the release of corticosteroid hormones from the adrenal cortex, which enter the blood stream and activates the sympathetic nervous system to produce an immediate 'fight-or-flight' response. In addition, corticosterone hormones cross the blood-brain-barrier and negatively regulate the production of CRH in the hypothalamus (Bali *et al.*, 2008). Thus, the hypothalamus controls the activation and inhibition of the stress response via a negative-feedback mechanism.

There are two major types of corticosteroid hormone receptors, the mineralocorticoid receptors (MCR), for which corticosteroids have high affinity, and glucocorticoid receptors (GCR), for which corticosteroids have a relatively low affinity. The high affinity for MCRs means that they are activated by the circadian fluctuation in basal levels and evidence supports MCRs as mediators of fast feedback and immediate responses, restoring homeostasis in seconds and minutes (De Kloet, 2003; Atkinson *et al.*, 2008). In contrast, GCRs are activated in response to significantly elevated levels of corticosteroids, such as in depression. The implications of GCR signalling are less understood, but current evidence suggests they mediate long-term responses and adaptations to corticosteroids, ensuring the system has increased tolerance to future stress exposure (De Kloet, 2003). This notion is in agreement with a potential neurogenic response that would ensure the plasticity of a hypothalamic network in order to maintain homeostasis long-term, for example in response to unpredictable and enduring temperature change. In order to investigate whether alpha-tanycytes, the neural stem/progenitor cell of the 3rd ventricle, are responsive to stressors I optimised a slice culture protocol for specific activation of GCRs (figure 7.4).

Western blots provide evidence of the expression of both GCRs and MCRs in the hypothalamus, and immunostaining reveals the expression of GCRs in the VZ and on Nestin-positive tanycytes (figure 7.4A). Dexamethasone (Dex) is a synthetic and specific ligand of the GCR. Addition of Dex to hypothalamic slices results in a concentration dependent increase in GCR expression, in agreement with the corticosterone-induced increase in GCR expression in-vivo and supporting 100μM Dex as a suitable model for stress induced GCR activation in hypothalamic slice cultures (figure 7.4B).

To investigate whether GCR activation led to a proliferative response from alpha-tanycytes, SC media was supplemented with 100μM Dex. In addition, as previous results provide evidence that Fgf signalling mediates alpha-tanycyte proliferation, Fgf receptor inhibitor, SU5402 was added to SC media alone or with the addition of Dex (figure 7.5). Addition of Dex significantly increases the number of PH3-positive alpha-tanycytes, supporting a proliferative response to GCR activation. This proliferative response was Fgf-dependent as co-culture of hypothalamic slices with Dex and SU5402 resulted in a significant decrease in PH3-positive cells compared to Dex alone. Culturing hypothalamic slices in the presence of GCR inhibitor, mifepristone, is not included in these results due to technical problems whereby it did not prove to be readily soluble. Despite this, these results provide significant evidence for a proliferative alpha-tanycyte response to GCR activation, suggesting high levels of corticosteroids through stressful situations influence hypothalamic progenitor proliferation in the adult. In addition, Fgf signalling is shown to be required for this effect, similarly to NMDA, indicating further that alpha-tanycyte proliferation in response to physiological stimuli is regulated by Fgfs.

Although proliferation is not a measure of neurogenesis, it is often a prerequisite to neuronal lineage progression. These data therefore suggest that alpha-tanycytes generate neural cell types in response to stress stimuli in order to maintain neural network plasticity and re-establish homeostasis via a long-term mechanism. Such a mechanism would prove valuable to an organism whose changing environment was not conducive to an immediate sympathetic response, for example where increased corticosteroids are produced due to diminishing resources, gradual restoration of the energy-balance via neurogenesis would reduce stress despite a lack of food, ensuring organism survival until resources are located. This model can be applied to all homeostatic functions of the hypothalamus. These hypothalamic slice cultures therefore focus future investigations by providing evidence of alpha-tanycyte activation in response to GCR activation, adding support to the use of transgenic lines to study adult hypothalamic neurogenesis in response to stress paradigms in-vivo, such as chronic mild stress as a model of depression (Schweizer *et al.*, 2009; Fonken *et al.*, 2009). In-vivo evidence that could support a neurogenic response to stress would be highly novel to the scientific community and of particular significance to contemporary society in which stress is an underlying factor of many pathologies, from hypertension to bipolar disorder.

In conclusion, the data presented in this chapter provide substantial evidence for Fgf-regulated proliferation of alpha-tanycytes, confirming and extending our previous in-vivo and in-vitro analyses. Furthermore, I provide evidence that alpha-tanycytes are responsive to excitotoxic levels of KA and NMDA, and suggest that alpha-tanycytes may be directly responsive to glutamatergic signalling, implicating a proliferative response to excitatory neurotransmission. In addition, the organotypic slice culture assay has proven invaluable in initial studies of the physiological significance of hypothalamic proliferation, supporting alpha-tanycytes as responsive to dexamethasone activation of GCR as a model for stress-induced activation. This response is Fgf-dependent and suggests that Fgf signalling is upregulated in response to GCR activation. This study provides strong foundations for future in-vivo analyses of a potential neurogenic response to stress hormones in the adult hypothalamus.

# Chapter 8

## Final discussion

## 8.1: Thesis overview

The results presented in this thesis have focused on distinguishing neural stem/progenitor characteristics of cells within the ventricular zone of the hypothalamic 3$^{rd}$ ventricle. In addition, an ex-vivo technique was developed and optimised for the study of the hypothalamic niche to elucidate its response to physiological stimuli.

Initially (chapter 3), I provided evidence of embryonic progenitor characteristics in the adult mouse ventromedial hypothalamus. Of note, high levels of expression of progenitor markers, including Sox3 and Six3, were found in alpha2-tanycytes, while Hes5, an effector of Notch signalling found in neural stem cells of the SGZ, showed restricted expression in the alpha2-tanycyte subtype. Alpha2-tanycytes also exhibit primary cilia and RC2-positive basal projections that are characteristic features of embryonic radial glia, the neural stem cells that populate the brain during development. BrdU retention assays provided evidence that alpha-tanycytes undergo division at E15/E16, retaining BrdU into adulthood. The combination of progenitor marker expression, embryonic radial glial characteristics, and thymidine retention supports alpha-tanycytes as slow-dividing neural stem/progenitor cells in the adult hypothalamus.

Inducing recombination of Glast::CreER$^{T2}$ mice leads to reporter expression in alpha-tanycytes specifically (chapter 4), within the 3$^{rd}$ ventricle ventricular zone (VZ), presenting an invaluable tool for interrogation of alpha-tanycytes as neural stem cells. Lineage tracing up to 9 months after recombination in unchallenged adult mice indicates alpha-tanycytes are multipotent in-vivo, generating tanycyte subtypes, astrocytes and sparse numbers of neurons. Furthermore, alpha2-tanycytes were found to be fibroblast growth factor (Fgf)-responsive, undergoing proliferation in response to FGF2 infusion. These studies identify alpha2-tanycytes as component cells of the hypothalamic niche, and support their neural stem cell identity in the adult.

Moving from in-vivo analyses to the in-vitro neurosphere assay presented an opportunity to ascertain the potential of different tanycyte subtypes to behave as neural stem cells, and further investigate the influence of Fgf signalling (chapter 5). Cells dissected from alpha2-tanycyte regions generated robust numbers of passageable neurospheres, while cells from beta-tanycyte regions could not. In addition, discrepancies were observed in passaging ability between ventral and dorsal alpha2-tanycyte populations, supporting heterogeneity in progenitor status. Fgf signalling was shown to be required for neurosphere formation, with neurospheres expressing endogenous *Fgf10* and *Fgf18,* that appear to differentially regulate neural stem cell maintenance and proliferation when added exogenously.

My identification of a niche and its regulatory factors, together with my strong evidence for alpha-tanycytes as the neural stem/progenitor cell, led me to elucidate their physiological role. Due to technical limitations of the neurosphere assay and the lack of time/resources to perform in-vivo analyses, I developed and optimised an organotypic slice culture of the tuberal hypothalamus for the study of proliferation in response to physiologically relevant stimuli (chapter 6). Importantly, collagen-embedded slices cultured in neurosphere medium without IGF/insulin maintained the three-dimensional structure around the 3$^{rd}$ ventricle and minimized proliferation. Organotypic slice culture, under these conditions, provided a tool to determine the proliferative responses of the different tanycyte subsets in a manner that recapitulates the in-vivo conditions.

Utilising the organotypic slice culture assay (chapter 7), I confirmed that Fgf signalling mediates alpha-tanycyte proliferation; in addition, I observed proliferation of alpha-tanycytes in response to kainic acid (KA)-induced excitotoxicity. Similarly, alpha-tanycytes proliferated in response to N-methyl-D-aspartate (NMDA); this proliferation, moreover, can be inhibited by Fgf receptor inhibition. The proliferative response to glutamatergic receptor activation by NMDA suggests that alpha-tanycytes are responsive to excitatory neurotransmission, a feature shared by neural stem cells in the classically-defined adult niches. As data supports alpha-tanycyte proliferation in response to neurotoxicity and neurotransmission, I added dexamethasone (Dex) to activate glucocorticoid receptors, modelling stress-induced physiological changes, a response regulated by the tuberal hypothalamus. In response to Dex-treatment of slice cultures, alpha-tanycytes were observed to undergo Fgf-dependent proliferation, suggesting that increased levels of stress activate hypothalamic adult neural stem/progenitor cells.

Together, these data provide novel evidence of (a) alpha-tanycytes as a neural stem/progenitor cell in the adult hypothalamus; (b) Fgf signalling as a crucial regulator of their activation, and (c) alpha-tanycytes as responsive to physiological stimuli. I will now discuss these results with respect to the findings I consider most significant, and how they are of value to the expanding field of adult neurogenesis research.

## 8.2: Identification of a neural stem/progenitor cell niche in the adult hypothalamus

Neurogenesis is primarily activated during embryogenesis when embryonic radial glial cells, descended from the neuroepithelium, undergo asymmetric cell division to generate neurons and populate the developing brain and spinal cord by tangential and radial expansion mechanisms (Stahl *et al.*, 2013; Lemke, 2009). Detailed analyses of the neural stem cells in the adult SVZ and SGZ have provided evidence that multipotent progenitors maintain embryonic radial glial characteristics, although neurogenesis in the adult is substantially diminished compared to that in the embryo. In the SVZ, B1-cells project a basal process, the end-feet of which extend onto the vasculature, and apically, project a primary cilia into the ventricle (Mirzadeh *et al.*, 2008). Similarly, SGZ neural stem cells have projections, which are orientated radially or horizontally (Lugert *et al.*, 2010), and require signalling through the primary cilia for their maintenance (Han *et al.*, 2008). In addition, classically-defined adult neural stem cells express embryonic progenitor markers, such as Sox2 (Brazel *et al.*, 2005a), and are regulated by factors that control neurodevelopment, including Fgfs (Kuhn *et al.*, 1997), Shh (Ahn and Joyner, 2005) and Notch (Ehm *et al.*, 2010). Such consistencies support the embryonic origins of neural stem cells found in the adult, strengthened by lineage tracing studies (Young *et al.*, 2007). Thus, to begin to address a potential neural stem cell niche in the hypothalamus in the context of the classically defined niches, I wished to identify embryonic characteristics of the 3rd ventricle ventricular zone (VZ) and its developmental origins.

Using a number of progenitor markers that label the VZ of the E15 mouse hypothalamus, I confirmed the maintained expression of embryonic neural progenitor markers in the adult hypothalamic VZ. Six3, a transcription factor restricted to the anterior neural tube, where it regulates proliferation and Shh transcription, is expressed in the VZ of the adult 3rd ventricle (figure 3.1). Of potential significance is the observation that alpha2 and beta1-tanycytes have higher levels of expression compared to alpha1 and beta2-tanycytes. Considering evidence that high levels of Six3 expression maintain embryonic forebrain progenitors in an undifferentiated state (Appolloni *et al.*, 2008), this supports the presence of undifferentiated neural progenitors in the alpha2 and beta1-tanycyte populations, that are potentially controlled by Shh-signalling. Similarly, Sox3, a transcription factor expressed by neural progenitors and required for the development of the hypothalamus, is expressed in the ventral 3rd ventricle (figure 3.2). Hes5, a transcription factor and downstream effector of canonical Notch signalling that is upregulated in undifferentiated cells (Basak and Taylor, 2007), shows remarkable restriction to ventral alpha2-tanycytes (figure 3.3). This is the first evidence that neural stem cell characteristics are maintained in the alpha2-tanycyte population alone, and provides an exciting target for future transgenic analyses.

Intriguingly, anteroposterior expression of Hes5::GFP reveals a changing pattern, that may provide an understanding of the changing morphology of the 3rd ventricle. While these are coronal sections, one can conceive that a transverse plane would show a ring of expression around the median eminence. Studies in chick have elucidated the contribution of anterior floor-plate cells to hypothalamic development and have provided evidence of Sox3-positive ‘collar cells’ that are regulated by Fgf signalling (Pearson *et al.*, 2011; Placzek and Pearson, 2013). The term ‘collar cell’ refers to the ring of floor-plate derived progenitors that encircle the developing infundibulum. The observation of a ring of Hes5::GFP expressing-cells, which are likely Sox3-positive (compare figures 3.3/3.4), in the ventromedial adult hypothalamus raises the fascinating prospect that these adult progenitors represent a maintained population of collar cells, and could therefore be direct descendants of the anterior floor-plate. A preserved progenitor population from early embryonic specification to old age might thus exist. This seems a reasonable idea, considering that development and maintenance of the hypothalamic-pituitary axis is vital to survival and reproduction; potentially, such a hypothalamic niche might be ancestrally older than the neurogenic niches in the dentate gyrus and striatum, whose contribution enhances chances of survival. Further investigation is required to build upon this hypothesis: in particular, the Hes5::CreER$^{T2}$ transgenic mouse (Lugert *et al.*, 2012) may present a valuable tool in identifying the relationship between the anterior floor-plate and adult alpha2-tanycytes.

Embryonic radial glia possess an apical primary cilia that projects into the ventricular space and a basal projection that spans radially to the cortical surface (Malatesta and Gotz, 2013). The preservation of this orientation is considered important for the correct progression through the cell-cycle, as the nucleus migrates to the apical membrane for mitosis and evidence implicates the apical-basal orientation of the mitotic spindle in determining symmetrical or asymmetrical division of neural progenitors (Postiglione *et al.*, 2011; Mirzadeh *et al.*, 2008; Rhyu and Knoblich, 1995). In addition, while basal projections have clearly-defined roles in neuronal migration, evidence supports their involvement in the orientation of the mitotic spindle and proliferation of neural progenitors (Kosodo and Huttner, 2009). Maintained polarity is clearly an important regulator of correct cell division, and as such, distinct features characterise it in radial glia. Recent studies have shown that the small GTPase, Arl13b, present in primary cilia, is required for the correct polarisation of radial glia (Higginbotham *et al.*, 2013). In addition, Arl13b is required for Shh signalling in primary cilia (Larkins *et al.*, 2011), known to be required for the formation of adult neural stem cells (Han *et al.*, 2008). My analyses provide evidence that alpha-tanycytes possess Arl13b-positive primary cilia, which are maintained on VZ cells from embryonic stages to adult (figure 3.6). In addition, RC2-positive basal projections are detected from the embryonic VZ and adult alpha2-tanycytes (figure 3.5), and are also expressed by radial glia at neonatal ages in the SVZ (Merkle *et al.*, 2004). These shared features further support alpha2-tanycytes as being under Shh regulation, and suggest a neural progenitor identity for which maintenance of polarity is crucial to regulate proliferation. Lineage-tracing embryonic radial glia in the hypothalamic VZ to adult stages will confirm or refute this idea; however the evidence that alpha2-tanycytes retain embryonic radial glia characteristics suggests an embryonic radial glial origin.

In support of the embryonic radial glial origin of alpha2-tanycytes, when BrdU is incorporated into the hypothalamic VZ of the 3rd ventricle in-utero, it is strongly retained in the alpha2-tanycyte region at adult stages (figure 3.8). While these data cannot rule out the possibility that BrdU retention is due to terminal differentiation, considering the progenitor characteristics of alpha2-tanycytes described, it is highly suggestive that a number of VZ cells incorporated BrdU before becoming quiescent/infrequently dividing. In order to confirm this notion, a second thymidine could be administered in adulthood to determine whether BrdU-positive tanycytes can re-enter the cell cycle, in-keeping with recent studies (Stoll *et al.*, 2011). Such studies should also be performed with a transgenic line, for example Hes5::GFP, to ensure that the double thymidine incorporation is a result of two rounds of cell division in the same cell, not cell division in a BrdU-positive daughter of a BrdU-positive cell. However, the observed low levels of proliferation in the adult hypothalamus, combined with the possibility that hypothalamic quiescent stem cells may act as a stem cell reserve in the niche (Lugert *et al.*, 2010), provides complications to the experimental approach. It may be more suitable to provoke proliferation of hypothalamic tanycytes and administer Ara-C to selectively kill proliferating cells. If the 3rd ventricle niche is recovered by a BrdU-retaining cell, or for example a Hes5::CreER$^{T2}$-positive cell, this would provide definitive evidence of a quiescent neural stem cell in the ventricular zone, as shown in the classically defined niches (Doetsch *et al.*, 1999a; Seri *et al.*, 2001).

I performed lineage tracing experiments in Glast::CreER$^{T2}$ mice to a maximum chase of 9 months in order to investigate the potential of tanycytes in adult mice. Glast::CreER$^{T2}$ is a marker of embryonic radial glial cells and adult neural stem/progenitor cells (Mori *et al.*, 2006; Ninkovic *et al.*, 2007). My acute analyses revealed that alpha-tanycytes specifically express the reporter after recombination (figure 4.2), while beta-tanycytes do not, allowing the first lineage tracing to be performed that is specific to the alpha-tanycytes: a significant distinction from previous hypothalamic progenitor lineage tracing studies. In addition, while previous hypothalamic progenitor lineage tracing studies had been performed in conjunction with energy-balance stressors, my study evaluates the potential of alpha-tanycytes in the unchallenged mouse, fundamental information in the continued characterisation of this region. Indeed, long-term chases provide evidence that alpha-tanycytes generate tanycytes, astrocytes and low numbers of neurons (figures 4.3-4.5). This novel result suggests that alpha-tanycytes are multipotent in-vivo. As this study has not been performed on single-labelled cells and their clones, it is possible that individual cells within the alpha-tanycyte population harbour different potential, and tanycytes (as progenitors) may be a completely heterogenous population. This would be consistent with a number of studies on the classically defined neural stem/progenitor niches (Giachino *et al.*, 2013; Young *et al.*, 2007; Bonaguidi *et al.*, 2011; Lugert *et al.*, 2010; Jhaveri *et al.*, 2012), and I encourage further detailed analyses at the clonal level in-vivo.

In-vitro, hypothalamic Glast::CreER$^{T2}$ cells are neurospherogenic. Careful subdissections of the 3rd ventricle into tanycyte subtypes revealed a minimum of three distinct neurospherogenic characteristics in alpha-tanycytes: alpha1-tanycytes generate low numbers of neurospheres that cannot be passaged further than 7° passage, ventral alpha2-tanycytes generate robust numbers that can not be passaged further than 4° passage, while dorsal alpha2-tanycytes generate robust numbers of neurospheres that can be serially passaged long-term (figure 3.2). These data provide further evidence of heterogeneity within and between tanycytes subtypes. Significantly, we have no evidence that beta-tanycytes are neurospherogenic, conflicting with a recent study that infers neural stem/progenitor cell characteristics in this subtype (Lee *et al.*, 2012a). The different conclusions from Lee and colleagues (2012) could be attributed to the juvenile mice studied, the use of a Nestin::CreER$^{T2}$ transgenic line that shows no tanycyte subtype specificity, the proximity of newborn cells in the median eminence to the beta-tanycyte subtype, and the influence of a high-fat-diet. Importantly, they provide no direct proof of neural stem cell characteristics in beta-tanycytes. In contrast, I show that alpha-tanycytes are multipotent in-vivo and neurospherogenic in-vitro.

Additionally, endogenous expression of *Fgf10* and *Fgf18* to the ventricular zone of beta- and alpha2-tanycytes has previously been shown, and their expression is maintained in hypothalamic neurospheres. Supplementing neurosphere media with these Fgfs results in apparent differences in the maintenance of cell-cycle dynamics, with Fgf18 promoting proliferation and Fgf10 reducing it (figure 5.4). Similar results were observed in organotypic slice cultures (figure 7.1). These results suggest that a balance of Fgf10 and Fgf18 controls the cell-cycle progression of a quiescent to actively proliferating progenitor. In support, inhibiting Fgf-signalling prevents neurosphere formation, while in the absence of exogenous Fgfs neurospheres are observed. These data support local Fgfs in regulating the activation status of the niche and may control the low levels of constitutive proliferation observed in-vivo (figure 4.3)(Kokoeva *et al.*, 2007).

Taken together, these data have identified a neural stem/progenitor cell niche in the adult hypothalamus. In addition, they suggest that alpha-tanycytes are a resident multipotent progenitor, and strongly suggest that cells within the alpha2-tanycyte subtype are remnant radial glial cells. In addition, they reveal that alpha2-tanycytes are neurospherogenic and proliferate in response to Fgf signalling. All the results support a significant level of heterogeneity in progenitor status between tanycyte subtypes and within subtypes, exemplified by varied levels of progenitor expression, neurospherogenic potential, response to Fgf signalling and neurogenic/gliogenic potential. These studies will be of significance in future studies as they provide clear evidence that alpha-tanycytes are component cells of the hypothalamic niche. The studies will focus future research into their regulation, contribution to neural circuitry and functional significance. Furthermore, while adult neural stem cell niches in mammals were previously considered to not exist, and subsequently thought only to exist in the SVZ and SGZ, these data confirm the presence of a neural stem cell niche in another discrete location, and therefore will contribute to the change in dogmatic principles surrounding the field of adult neurogenesis.

## 8.3: Role of alpha-tanycytes as progenitors in the adult hypothalamus

Long-term lineage tracing of alpha-tanycytes gives evidence of the generation of three distinct neural cell types in the unchallenged mouse. Over time, reporter expression spreads ventrally to beta1-tanycytes and dorsally to alpha1-tanycytes and ependymocyte populations (figure 4.3). These reporter-positive cell types may represent differentiated cells that have been generated to replace old or damaged VZ cells. Ciliated ependymocytes are essential in the propulsion of CSF through the ventricles, and are considered important in filtering harmful substances and cellular debris away from brain tissue, and are derived from embryonic radial glia (Spassky *et al.*, 2005). Alpha-tanycytes may elicit the turnover of ependymocytes to ensure the 3rd ventricular ependymal layer integrity is maintained throughout the life of an organism.

Tanycytes act as conduits and barrier cells, ensuring brain-body communication while controlling absorption/secretion of factors across the circumventricular organ, which lacks the classical blood-brain barrier (Rodriguez *et al.*, 2005; Mullier *et al.*, 2010). In addition, they have a central role in neuroendocrine release, including physically regulating gonadotropin releasing hormone (GnRH) release and thus subsequent sexual maturation, ovulation, pregnancy and parturition (Prevot *et al.*, 1999; Prevot *et al.*, 2010b). Considering the significant role of tanycytes in correct hypothalamic function, and the required role of the hypothalamus in maintaining homeostasis of systems that are crucial to survival of the organism and species, the presence of a neural stem/progenitor cell type that can regulate tissue homeostasis at the ventricular zone would be a beneficial feature. Evidence to support this comes from the spread of reporter-positive cells at the ventricular zone over time in Glast::CreER$^{T2}$ mice, and additionally from BrdU retention studies in which an increase in VZ BrdU-positive cells is observed and maintained in pubescent female mice, onwards (figure 3.8).

These data support the role of alpha-tanycytes in maintaining differentiated tanycyte populations. The lack of a blood-brain barrier at the median eminence could leave tanycytes vulnerable to cell death by toxins, pharmaceutical agents and infection; thus production of tanycytes from the alpha-tanycyte population may be a crucial mechanism to maintain integrity of this circumventricular organ as a gatekeeper to the central nervous system. Indeed, when alloxan is administered to destroy Gfap-positive tanycytes, a recovery is observed after two weeks (Sanders *et al.*, 2004), supporting a local progenitor population. Such features could be shared in other circumventricular organs, such as the organum vasculusom of the lamina terminalis and the area postrema. In accord, both these areas have been shown to express neural stem cell markers, and are neurospherogenic and multipotent in-vitro (Bennett *et al.*, 2009).

In addition to the generation of differentiated VZ cell types, the increase in reporter-positive tanycyte subtypes that have neural progenitor characteristics suggests a number of points. Firstly, the increase in density of alpha2-tanycyte reporter-positive cells supports the self-renewal of alpha2-tanycytes, maintaining the neural stem/progenitor pool. Secondly, the increase in reporter-positive alpha1-tanycytes may represent a population of lineage-committed progenitors, supported by their limited neurospherogenic potential. Similarly, the observation of reporter-positive beta1-tanycytes over time may represent lineage-committed progenitors, reported to have neurogenic capacity (Lee *et al.*, 2012a), and suggest that these are derived from alpha2-tanycytes. In order to differentiate between progenitors and differentiated tanycytes, future studies could assess the mitotic status of alpha2-tanycyte progeny to determine if they are post-mitotic differentiated cells or cell-cycling progenitors. In the unchallenged mouse, the Glast::CreER$^{T2}$-positive population of alpha-tanycytes generates VZ-cells, likely maintaining tissue homeostasis of differentiated tanycytes, progenitor pools and the protection of the circumventricular organ.

In addition to the reporter-positive Gfap-positive cells observed in the VZ after a 9-month chase, a similar proportion is observed in the parenchyma (figure 4.5). A significant increase in the number of parenchymal reporter-positive Gfap-positive cells over time provides strong evidence that Glast::CreER$^{T2}$-positive tanycytes generate astrocytes. Traditionally, many studies attribute more importance to the observation of neurogenesis than gliogenesis. This is understandable as the adult brain permits gliogenesis, while neurogenesis is maintained in discrete locations, and thus gliogenesis is not necessarily considered novel. However, care must be taken in distinguishing a novel result and the significance of a result.

The production of astrocytes, i.e. the continued gliogenesis, from alpha-tanycytes could be extremely important in the adult hypothalamus. Astrocytes have established neuroprotective roles, including the provision of nutrients, sequestration of excess neurotransmitters and regulation of ion balance. These features are likely to be important in the hypothalamus where maintained neuronal circuitry is important for physiological homeostasis. Beyond this, the role of astrocytes is unclear, although evidence suggests their role is not limited to neuroprotection. Recently, human astroglial progenitors were transplanted into postnatal mouse brains, resulting in enhanced synaptic plasticity and improved learning that was attributed to the human-like astrocytes (Han *et al.*, 2013). These data suggest that human astrocytes regulate long-term potentiation, and while human astrocytes are more complex than murine astrocytes, their roles may be shared. Conceivably, then, astrocyte production from alpha-tanycytes may ensure flexibility of homeostatic circuitry.

In contrast to the relatively high numbers of VZ cell and astrocytes produced, neurons are observed at low frequency (figure 4.4). However, the presence of reporter-positive immature neurons close to the alpha-tanycyte region in 6 week chased animals, and mature neurons in arcuate and ventromedial nuclei after 9 months, is evidence that alpha-tanycytes have the potential to generate neurons in the unchallenged mice. The role of alpha-tanycytes may therefore be, as in the SVZ (Nissant *et al.*, 2009) and the SGZ (Van Praag *et al.*, 1999), to generate newborn neurons that support increased synaptic plasticity. Addition of newborn neurons could lead to a refinement of neural circuitry to reinstate and/or maintain homeostasis in the context of a challenge. Additionally, the alpha-tanycytes may generate neurons to replace old or damaged neurons and ensure homeostasis is maintained. In this study, it seemed likely that the mice were not challenged by environmental factors, having a regulated environmental temperature, light cycle and access to food; therefore the observed neurogenesis may be to replace dying neurons. Indeed, slice culture assays support an alpha-tanycyte response to neuronal death (figure 7.2), although the experimental approach did not allow the long-term tracing of alpha-tanycytes that would prove a neurogenic response.

Alternatively, a factor to consider in the lineage tracing of alpha-tanycytes is that although we assume the housing conditions do not challenge mice, the close proximity of individuals to others, the presence of animal-facility staff and the caging of mice in an environment that they did not evolve to thrive in, may itself be a challenge. Furthermore, early life experiences can have significant consequences on an organism's sensitivity to challenges. In agreement, studies of rat pups shows that rearing in different bedding types leads to differences in anxiety behaviours (Sakhai *et al.*, 2013), while maternal licking and grooming has a dramatic influence on offspring's sensitivity to stress later in life (Champagne *et al.*, 2008). As we did not record maternal licking or bedding, the observed neurogenesis could be a response to this; however as mice are raised in this consistent environment, it is unlikely that neurogenesis is required in adulthood to change and refine neural circuitry. To address this, future studies could house wild mice in controlled conditions, or follow laboratory-housed mice in wild conditions to identify homeostatic discrepancies between environments. Yet, studies such as this will have inherent animal-welfare and animal tracking issues.

Nevertheless, the data provided in this thesis provide evidence that a population of alpha-tanycytes have neurogenic potential. Significantly, the neurosphere assay, organotypic slice culture and in-vivo infusions all provide evidence that alpha-tanycyte proliferation is regulated by Fgf signalling. Research into the classically-defined neurogenic niches has identified Fgf signalling as an important regulator of neurogenesis (see section 1.1.2,1.1.3). In the SVZ, Fgf2 signalling increases the production of neurons at the expense of astrocytes (Kuhn *et al.*, 1997). While infusion of exogenous Fgf2 has little effect on the generation of newborn neurons in the dentate gyrus (Kuhn *et al.*, 1997), SGZ neurogenesis in response to brain injuries is mediated by Fgf2 (Yoshimura *et al.*, 2001; Yoshimura *et al.*, 2003). If the role of Fgf signalling were conserved in the hypothalamic neural stem/progenitor cell niche, then the proliferative response to Fgf2 (figures 4.6, 6.6) would be indicative of an increase in neurogenesis. My Observations that alpha-tanycytes proliferate in response to neuroexcitotoxicity (figure 7.2) would, therefore, also be consistent with studies that identify an increase in Fgf2-dependent SGZ neurogenesis in response to kainic acid-induced seizures (Yoshimura *et al.*, 2001; Lugert *et al.*, 2010). Future studies should utilise transgenic mouse lines, such as Glast::CreER$^{T2}$ or Hes5::CreER$^{T2}$, to assess whether alpha-tanycyte neurogenesis is increased in response to Fgf2, and the extent to which Fgf mediates a hypothalamic neurogenic response to brain injury models and physiological stressors.

My studies provide evidence that alpha-tanycytes have the potential to generate tanycytes, astrocytes and neurons in-vivo, and my data supports their self-renewal capacity. In addition, the organotypic slice culture assays provide further support to alpha-tanycyte proliferation being Fgf regulated, inhibition of signalling reducing the proliferative response to ionotropic glutamate receptor activation and glucocorticoid receptor activation. The significance of newborn cell generation from alpha-tanycytes has not been studied, but it is reasonable to believe they would provide a vital function in tissue homeostasis, including replacing damaged VZ cells and neurons, and in refining neural networks by addition of astrocytes and neurons to support flexibility. This study will be of importance to future studies that assess the physiological significance of the hypothalamic niche, and studies that address differences in neurogenic potential of cells within the highly heterogenous alpha-tanycyte subtype.

## 8.4: Functional implications of alpha-tanycytes as progenitors in the adult hypothalamus

Glutamatergic signalling activation via glutamate is the major method of excitatory neurotransmission, resulting in cation influx, depolarisation of the neuron and an action potential that can be propagated to the next presynaptic terminal. Activation of the ionotropic glutamate receptor, NMDA receptor, is considered to mediate long-term potentiation and synaptic plasticity, important in learning and key features of newborn neurons (Nacher and McEwen, 2006). Concurrently, the regulation of neural stem cells in response to excitatory and inhibitory neurotransmission has been concluded in both the SVZ and SGZ (Song *et al.*, 2012; Brazel *et al.*, 2005b).

Overstimulation of glutamate receptors results in excitotoxicity, the mechanism whereby high levels of calcium ions flow into neurons and lead to a cascade of cell degeneration, ultimately resulting in neuronal death. A number of studies have demonstrated increased proliferation and neurogenesis in adult niches in response to excitotoxic stimuli (Aguado *et al.*, 2007; Lugert *et al.*, 2010); further, overstimulation of glutamate receptors is known to cause neuronal death in the hypothalamus (Katsuki and Akaike, 2004). In agreement, treatment of organotypic hypothalamic slice cultures with high concentrations of kainic acid, a pharmacological agonist of an ionotropic glutamate receptor, leads to alpha-tanycyte proliferation (figure 7.2). Similarly, NMDA receptor overstimulation leads to proliferation in the alpha-tanycyte subregion (figure 7.3). Furthermore, a maximal proliferative response is observed at the lowest concentration of NMDA used, which could suggest alpha-tanycytes proliferate in response to excitatory neurotransmission in the absence of excitotoxicity. The expression of glutamate-aspartate transporter (Glast) on alpha-tanycytes specifically, within the 3rd ventricle VZ (figure 4.2), adds support to the notion that hypothalamic neural stem/progenitor cells are directly sensitive to excitatory amino acid levels.

Ionotropic glutamate receptor activation, and therefore excitatory neurotransmission, can be considered to be a response to physiological stimuli. The proliferative response of alpha-tanycytes to NMDA in slice culture, and in the neurosphere assay at a lower concentration (figure 5.5), supports an alpha-tanycyte response to physiological stimuli. To date, most studies have focused on the impact of light period (Shearer *et al.,* 2012) or energy homeostasis in the physiological control of hypothalamic progenitors (Lee *et al.*, 2012a; Haan *et al.*, 2013; McNay *et al.*, 2012; Kokoeva *et al.*, 2005). However, the hypothalamus is the central regulator of the stress response, activating glucocorticoid release from the adrenal gland to redistribute energy accordingly and then subsequently inactivating the response, thereby maintaining physiological homeostasis (Herman, 2013). Significantly, NMDA receptor activation is implicated in an organisms' sensitivity to stress. Adult male rats that receive high levels of maternal-licking and grooming exhibit increased synaptic plasticity in the dentate gyrus and enhanced learning, an effect that is reversed in stressful environments (Champagne *et al.*, 2008). In contrast, while offspring from low-licking mothers show less learning under basal conditions, in response to stress they exhibit enhanced synaptic plasticity and learning. The mechanism for this context-specific maternal programming is, in-part, mediated through NMDA receptor activation that mediates long-term potentiation; offspring of low-lickers have basally elevated NMDA receptor function that is not affected by stress hormone administration, while the converse is true in the offspring of high-lickers (Bagot *et al.*, 2012). Considering the role of NMDA receptor function in synaptic plasticity and stress, and the central role for the hypothalamus in restoring homeostasis in response to stress, the NMDA induced proliferation of alpha-tanycytes may reflect a physiological response to stress.

Hypothalamic-pituitary-adrenal axis activation by stressors, either physiological or psychological, results in glucocorticoid release into the blood, mediating the stress response. It is important to note that glucocorticoids are also released in circadian rhythms in the absence of 'stress', with mineralocorticoid receptors being activated by such basal levels (Herman, 2013). These basal levels are important for recruiting energy when it is most needed during waking hours, and studies suggest the immediate sympathetic response to a stressor is also regulated by mineralocorticoid receptors (Atkinson *et al.*, 2008). As Shearer and colleagues (2012) have shown a discrepancy in proliferative response associated with the length of daylight exposure, in the region of alpha-tanycytes in rat; long-term photoperiod changes and short-term diurnal rhythms, like that of glucocorticoid release, may influence adult hypothalamic plasticity. It is intriguing to consider that Fgf10 and Fgf18 may be differentially upregulated during the circadian cycle, where changes in mineralocorticoid receptor activation could stimulate Fgf-dependent proliferation, maintaining fine-control over daily and seasonal tissue homeostasis (Dickmeis *et al.*, 2013). This notion could begin to explain the observed in-vitro influence of Fgf10 and Fgf18 upon cell-cycling, and implicates the importance of their balance in the routine maintenance of hypothalamic networks.

Glucocorticoid receptors have a much lower affinity for glucocorticoids, and are thus activated in response to high levels of stress, and are considered to regulate the adaptive response to stress (De Kloet, 2003). The adaptive response to stress ensures biological efficiency and internal homeostasis, including glucose and insulin regulation, immune regulation and cellular maintenance. Activation of glucocorticoid and mineralocorticoid receptors by glucocorticoids results in an upregulation of receptors, which ensures the tissue is more receptive to stress and that subsequent exposure to high levels of glucocorticoids does not cause neuronal death. In the paraventricular nucleus of the hypothalamus, the projection target of alpha1-tanycytes, glucocorticoid receptor activation inhibits further activation of the stress response, and ensures a return to homeostasis (Herman, 2013). With the aid of inputs from the amygdala, prefrontal cortex and hippocampus, the hypothalamus maintains an adaptive response to glucocorticoids, fundamental to survival. However, models of human depression using chronic, non-habituating, mild stress in rodents, eventually leads to a maladaptive response, whereby the normal stress response occurs out of context (of a stressful situation) and no longer confers a survival advantage (Herman, 2013). This can be considered to be a relatively recent dysregulation, as our modern lifestyles contrast to the environment in which humans evolved.

Glucocorticoids upregulate Fgf2 expression throughout the central nervous system, and similarly, acute and chronic stress upregulates Fgf2 expression (Molteni *et al.*, 2001). Indeed, research has shown that the stress-induced increase in Fgf2 expression is mediated by glucocorticoids (Frank *et al.*, 2007; Meisinger *et al.*, 1996). In the hypothalamus, I have shown that Fgf receptor inhibition prevents the proliferative response of alpha-tanycytes to dexamethasone, a pharmacological glucocorticoid receptor agonist (figure 7.5). This result suggests that hypothalamic neural stem/progenitor cells respond to increased levels of stress. In addition, IGF-1 stimulates alpha-tanycyte proliferation (Perez-Martin *et al.*, 2010), and mediates resilience to stress in enriched environments (Baldini *et al.*, 2013). These data support future investigation into the neurogenic response of alpha-tanycytes to stressful stimuli, where newborn neurons may contribute to the adaptive stress response.

Importantly, stress and glucocorticoid receptor activation have been extensively researched in the dentate gyrus, where both are observed to decrease proliferation and neurogenesis (Mirescu and Gould, 2006). For this reason, glucocorticoids are widely considered to have negative effects on neurogenesis as a whole, despite there being no change in SVZ proliferation in response to multiple chronic mild stressors (Schoenfeld and Gould, 2013). Indeed, while many reports conclude that SGZ neurogenesis is suppressed in response to stress (Gould and Tanapat, 1999), this is conflicted by reports in which glucocorticoid receptor activation is shown to be required for newborn neuron survival (Snyder *et al.*, 2009) and correct integration (Fitzsimons *et al.*, 2012). Indeed, running enhances glucocorticoid levels and neurogenesis; therefore the neurogenic response to stress is heavily dependent on context (Snyder *et al.*, 2009; Schoenfeld and Gould, 2012). In the context of chronic mild stress, a reduction in hippocampal neurogenesis may well be adaptive, re-routing energy away from learning and memory functions to regions that confer survival. Indeed, the tendency for pattern completion in the absence of SGZ neurogenesis (section 1.1.4, page 43) could confer a survival advantage in itself, as dangers/stressors will be grouped to elicit the fight-or-flight response, compared to pattern separation in which dangers/stressors would stimulate an individual response. Thus, a fox-shaped object may stimulate flight in a rat (even if it was not a fox) and decrease its chance of predation.

While glucocorticoids may downregulate proliferation in the dentate gyrus in the absence of a rewarding experience (Schoenfeld and Gould, 2013), this does not necessarily mean they downregulate proliferation in other cell types. Indeed, stress-induced elevation of glucocorticoids in microglia, the macrophages of the central nervous system, increases proliferation through an NMDA receptor-dependent mechanism (Nair and Bonneau, 2006). Proliferation in this context may ameliorate phagocytosis of damaged neurons, while as in other systems, chronic stress could result in maladaption: increased brain inflammation and phagocytosis of neural cells.

Fgf signalling regulates alpha-tanycyte proliferation in response to dexamethasone, and is globally upregulated in response to stress and glucocorticoid activation. This, combined with the observation that NMDA receptor activation can similarly increase alpha-tanycyte proliferation, and mediates synaptic plasticity and stress sensitivity, leads me to hypothesise that stress and glucocorticoids activate hypothalamic progenitors in order to provide flexibility to the vital stress adaptation response. Seasonal changes in light, temperature, resources and reproductive status would be maintained by hypothalamic circuitry that is underpinned by alpha-tanycytes, generating new tanycytes, astrocytes and neurons to replace and refine homeostatic machinery. However, as in other regions of the central nervous system, the adaptive response of alpha-tanycytes to stress may become maladaptive, resulting in hypothalamic network changes that are not conducive to survival, such as weight gain, diabetes, depression, amenorrhea; all side effects of dexamethasone treatment (NHS choices, 2013). My organotypic slice cultures therefore provide the basis for further investigation with transgenic mouse lines to determine the alpha-tanycyte response to stressors in-vivo.

## 8.5: Concluding remarks

My thesis provides evidence for a multipotent neural stem/progenitor population within the alpha2-tanycyte population of the adult hypothalamus, supporting this cell type as a vital component cell of a newly identified niche. I provide evidence of the embryonic origins of alpha2-tanycytes, and their shared features with embryonic radial glia. My long-term lineage tracing of alpha-tanycytes suggests their multipotent potential in-vivo in the unchallenged mice, a view which is confirmed and extended in-vitro through the neurosphere assay. In addition, I have developed and optimised an organotypic slice culture assay that I utilised to begin to investigate the factors that regulate alpha-tanycyte proliferation. I confirmed that Fgf signaling mediates alpha-tanycyte proliferation in-vivo, ex-vivo and in-vitro. In addition, I provide the foundations for future investigation into the alpha-tanycyte response to stress, suggesting a role in maintaining tissue homeostasis and physiological homeostasis. These data advance the field of adult neurogenesis, and provide conclusive evidence of a heterogenous population of neural progenitors that are responsive to Fgf. My work forms the basis of future investigations that will examine a novel role for glucocorticoids in activating proliferation, and potentially neurogenesis, in alpha-tanycytes.

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